ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME 5

1951

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA, U.S.A.

84099

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA, U.S.A.

FOREIGN AGENCIES

H. K. Lewis & Company, Limited
136 Gower Street
London, W.C. 1

Mezhdunarodnaya Kniga
Kuznetsky Most, 18
Moscow

Martinus Nijhoff
9, Lange Voorhout
The Hague

Maruzen Company, Limited
6, Tori-Nichome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA
BY GEORGE BANTA PUBLISHING COMPANY

PREFACE

The Preface to this *Review* provides another opportunity to express our gratitude to those who have written the chapters for this volume. Their surveys of advances in various fields of microbiology are important contributions to growth in this field of science. The Editorial Committee continues to welcome suggestions both for topics and authors and for improvements of the *Annual Review of Microbiology*.

The problems of inclusion of titles and of arrangements of entries in the bibliographies remain controversial. Annual Reviews, Inc., has published its views on these matters in the Preface to Volume 20 of the *Annual Review of Biochemistry*. The most important consideration is that publication and distribution costs necessitate condensation. It has been deemed desirable to achieve this by the use of smaller type rather than by decreasing coverage.

We repret that limitations of time have necessitated postponement for one year of the publication of the review on Nutrition and Metabolism of Spirochetes by Q. M. Geiman.

We extend once again our appreciation for the valuable assistance and hearty co-operation of the Annual Reviews' office staff and for the care exercised by the George Banta Publishing Company in the preparation of this volume.

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page 256, lines 30 and 31: for *Neurospora sitophial* read *Neurospora sitophila*

page 260, reference 105: for *Phytopathology*, **37,** 847 (1947) read *Phytopathology*, **40,** 23 (1950)

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THE MORPHOLOGY AND CYTOLOGY OF BACTERIA[1]

By K. A. Bisset

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Introduction

I have been told that a very eminent, American cyto-geneticist recently remarked, when speaking of bacterial cytology, that there are two stages in the progress of a scientific problem; the first, in which the findings are highly improbable, and the second, in which they are obvious. It must honestly be admitted, however, that the contemporary synthesis of bacterial cytology, although now embarking on the second stage, has abandoned much which was once claimed to be true, although embodying much more which has been accurately reported, at intervals, over the last sixty years, but almost entirely ignored by the majority of bacteriologists. It is not the purpose of this review, nor is it the policy of this journal to discuss in detail these early studies, but rather to correlate the most recent work, in the hope that no defence of the subject is now required.

Some recent discoveries have been made possible by advances and improvements in technique, others by the careful application of known methods to new problems, or even to the reinvestigation of old ones. Perhaps the greatest forward step has been the attainment of a sufficient body of mutually corroborative information, both cytological and genetical, to enable a general system to be formulated, within the framework of which further observations, not only those made subsequently, but others also, already known but difficult of interpretation, may be given some degree of systematic order.

In general, it appears that the resting stages of bacteria, whether these be endospores [Robinow (101), Bisset (16)], the resting cells of non-sporing bacteria [Bisset (15, 18)], microcysts of myxobacteria [Klieneberger-Nobel (61)], spores of *Streptomyces* [Klieneberger-Nobel (60)], or cysts of *Azotobacter* [Pochon, Tchan & Wang (96)], possess a typical, vesicular nucleus, often staining eccentrically, but in most respects quite similar to the nuclei of other small cells. In bacteria whose resting stages are sufficiently large, the structure of this nucleus may quite easily be seen; that of the resting stage of the typhoid bacterium has a well-marked nuclear membrane and an eccentric chromatinic granule [Bisset (19)]. Its resemblance to the nucleus of certain yeasts [Lindegren (69)] is so close as to be quite startling.

The mitotic figures of bacteria also resemble those of yeasts more closely than they do those of plants and animals. The paired nucleoids divide reductionally [Bisset (11, 17)] and, hence, cannot be regarded as true chromosome pairs. The general appearance of these comparatively massive

[1] The survey of the literature pertaining to this review was concluded in January, 1951.

bodies is much more suggestive of the chromosome complexes of dividing yeasts [Lindegren (69)] than of conventional chromosomes. These chromosome complexes, as they appear in young cultures of bacteria, are now fairly well recognised by bacteriologists. They were first demonstrated in a convincing manner by Robinow (99, 100, 101) and have been described by many other authors. Robinow's demonstration, like his method, was an exceedingly skilful exposition of facts which had already been recorded, but not previously accepted. The staining technique which produced this revolution of thought was essentially a modification of the Feulgen reaction, and was employed for the same purpose by Piekarski (93). An essentially similar technique was used by Gutstein (48) in a cytochemical analysis of bacterial and other nuclei. The basic principle is mild acid-hydrolysis, which renders stainable the desoxyribose nucleic acids of the nucleus, also reducing the affinity for dyes of the ribose nucleic acids in the surface layers of the cytoplasm, which mask the internal structures in untreated cells.

The much greater difficulty of staining the nuclear structures in cells from cultures more than a few hours old, together with the fact that Robinow (101) stated that, after a few hours, the nuclear material was diffused throughout the cell, resulted in the details of the nuclear cycle in nonsporing eubacteria remaining unnoticed for several years, while the comparable phenomena in sporing bacilli [Klieneberger-Nobel (59); Flewett (43); Wang & Tchan (115)], and in myxobacteria [Beebe (5); Klieneberger-Nobel (61)] were fully investigated. It eventually transpired, however, that the process of maturation of the resting cell of non-sporing eubacteria [Bisset (15, 17)] is very similar to that of the endospore or the myxobacterial microcyst. The reductional mode of division of the chromosome complexes [Bisset (11, 17)] and the reduction process immediately following the nuclear fusion in the process of maturation of these several types of resting cell [Klieneberger-Nobel (60, 61); Grace (45); Bisset, Grace & Morris (22)], provide a picture which is well correlated with the genetical evidence of Lederberg (68). Once more the closest resemblance is with those yeasts which are normally haploid, and in which copulation is followed immediately by reduction and restoration of the haploid condition. Observations suggestive of sexual conjugation in bacteria, which have frequently been reported, have been reinforced by these genetical studies, which go far to prove that such appearances do, in fact, indicate sexual processes. The author has recorded and assessed the evidence for sexuality in bacteria, at some length, in a recent monograph [Bisset (17)], and is of the opinion that the evidence is strongly in favour of its occurrence.

Another striking advance in our knowledge of bacteria is the recognition of the fact that many forms are multicellular. This is true of most large, sporing bacilli [Robinow (101)], of corynebacteria and mycobacteria [Bisset (14)], of lactobacilli [Bisset (10)], of the large bacterium *Caryophanon* [Pringsheim & Robinow (92)], and even of gram-positive cocci [Bisset (9, 13)]. Even those gram-negative, intestinal bacteria which are normally unicellular, may produce multicellular forms in young cultures [Robinow

(101). Those, such as the intestinal bacteria and streptococci, which may adopt either the "rough" or "smooth" morphology, may accordingly exist in a unicellular or multicellular phase, the "rough " form possessing a curious, symmetrical type of multicellularity, which is shared by the chain-forming sporing bacilli and the lactobacilli [Bisset (8)]. The structure of the rough colony is due, of course, to this chain-forming habit of growth, and is typified in the "Medusa-head" colony of *Bacillus anthracis*. Failure to recognise this multicellularity has led several workers to describe multiple nuclei in bacteria which were, in fact, multicellular, with a single nucleus in each cell. This applies to the descriptions of *Caryophanon* by Peshkoff (83), of *Mycobacterium tuberculosis* by Brieger & Robinow (28), and of certain stages in the life-cycle of *B. anthracis* by Flewett (43). A recent and lengthy paper by Knaysi, Hillier & Fabricant (66) shows that these workers have also missed the significance of this important point, as applied to mycobacteria.

The majority of recent cytological papers have been published in English, and as a natural result, very full reviews have been published in other languages. Among the best are those of Piekarski (95) in German, and Parvis (80) and Leonardi (70) in Italian.

Recent Advances in Observations and Techniques

Although considerable advances have been made in techniques applicable to the study of bacterial cytology in recent years, it is remarkable that these have almost invariably served merely to confirm and extend observations already recorded (if not always well known), which were made by classical methods. In some cases the revision of these observations has been so extensive as to lend them a new complexion: this is true of the studies of Klieneberger-Nobel (59) and others upon the cytology of sporulation, which confirmed many of Schaudinn's well-known observations, but gave them a new and more lucid background. Here as elsewhere phase-contrast microscopy [Pulvertaft (97)] has confirmed the inferences drawn from fixed and stained material. Observations upon the process of sporulation by electron microscopy have provided little or no information.

Confirmation of the behaviour of the nuclear bodies in dividing bacteria has also been provided by phase-contrast microscopy (Tulasne (112); Stempen (109). Boyd (26, 27) has made observations upon the cytology of phage infected bacteria, and Haselmann & Kappel (50) have made them upon gonococci, by the same method. It is characteristic of these attempts at high-magnification, phase-contrast microscopy, and also of the author's own experience with the technique, that the excellent contrast is not matched by a corresponding excellence of definition. The latter in fact, could be greatly improved, as no doubt it will be, in the near future.

In addition to numerous studies performed by methods which are now classical, modifications of these techniques have been invented. These do not require detailed comment; Cassel (31) employed cold perchloric acid in place of hot hydrochloric acid in the preliminary hydrolysis procedure of the

acid-Giemsa technique; Smith (106) recommended fixation in formaldehyde after hydrolysis and staining with basic fuchsin instead of Giemsa. The latter is a return to an older method [Gutstein (48)] in some respects. Pennington (82) has also recorded the effect of formaldehyde upon the stainability of bacteria.

Several workers have attempted to elucidate the problem of bipolar staining and metachromatic, polar granules [which are quite distinct, although often confused, see Bisset (17)]. Wei, Tchan & Pochon (117) investigated the polar granules in *Pasteurella* and *Pfeifferella* and recorded the conclusion that they were the normal, nuclear bodies in this type of cell. Wachstein & Pisano (113) devised a new method for staining the polar granules of *Corynebacterium diphtheriae* and other bacteria with lead salts; and Oeding (77) investigated their staining reactions after subjection to hot water. Both claimed that the bodies failed to stain by Feulgen's method, an observation with which the author profoundly disagrees, and Oeding stated that the granules were confirmed to be composed of "volutin," which term, like most of its users, he failed to define. The author has shown [Bisset (14)] that the polar bodies of corynebacteria are the shrunken contents of the terminal cells of these multicellular bacteria, and contain both nuclear and cytoplasmic material. They are not properly seen in preparations which have been spared the shrinkage inseparable from heat fixation. The phenomenon of bipolar staining, as the term is usually applied to unicellular bacteria, is an optical illusion and is discussed under IMSENECKI'S ARTIFACT THEORY.

It is rather surprising to find that many bacteria actually show unipolar staining. That is to say, they have an active growing-point at one end of the cell, which stains more strongly than the other, because of the high concentration of nucleoproteins, mainly engaged in the secretion of the cell wall [Bisset (20)].

General investigations of bacterial morphology have been made by cytochemical staining methods. Burdon (30) described fatty structures in many species, and Pennington (81) and Jarvi & Levanto (55) published similar general descriptions of polysaccharide structures in bacterial cells, by the use of periodic acid and silver nitrate. These investigations were not productive of any outstanding cytological discoveries, but emphasised the structural complexity of many bacteria, which is still liable to be overlooked. Both techniques served to demonstrate the multicellularity of certain species, by staining the lipo-protein cell membrane and the polysaccharide cell wall respectively. Lamanna & Mallette (67) investigated the nucleic acids of the cell wall.

THE FLAGELLAR CONTROVERSY

The theory of Pijper, published in 1946 (86), and subsequently republished, in essentially the same form, in different journals (87, 88), that bac-

terial flagella are artifacts, "twirls" of polysaccharide, drawn out from the surface slime layer by the spiral movement of the cell, has attracted a considerable amount of attention. Although almost all experimental work by other people supports the classical view of the nature of flagella, and these authors have been very critical of Pijper's views, a number of general surveys and text-books have been lenient, even favourable to Pijper in the discussion of this point. This appears to have caused confusion in the minds of those who, being themselves unfamiliar with the problem, depend upon such commentaries for their information. It is my opinion that Pijper's theory is completely untenable, and that even those who have so far contested it have failed to expose sufficiently rigorously the falsity of the logic by which his conclusions are derived from his evidence.

It is not difficult to understand how these theories evolved. Pijper's original observations upon the motility of typhoid bacteria were made in the course of his studies of flagellar agglutination, and led him to the belief that the flagella, as usually seen in stained preparations, or with the electron microscope, are elementary strands of a complex tail, which is the main organ of propulsion (85). Later studies, by the same technique of dark-ground cinematography, in which he concentrated more closely upon the body of the bacterium, showed that the motion of the swimming cells was spiral (not in itself an original observation) and that the shape of the cells was also slightly spiral (86). On this evidence, almost exclusively, Pijper changed his opinion, and adopted the theory that the tail was not an organ, but was the result of the twisting together of the elementary flagella. This was probably correct, but Pijper's further conclusion that the tail took no part in propulsion, but was dragged passively behind the cell, does not follow from this observation, and seems indeed, from my own study of the motion pictures upon which it was based, to be highly conjectural. His deduction from this doubtful assumption, that not only the tail, but all flagella are mere polysaccharide "twirls," drawn out from the slime layer, is entirely unjustifiable. Even if it could be taken as proved (which it cannot) that the tails are mere passive appendages, this further conclusion ignores the alternative explanation that the motility of the cell is due to flagella, not included in the tail, and unresolved by visible light. By analogy with those water bacteria which swim so actively by means of a single flagellum, no great number of free flagella would be needed to produce this effect.

Pijper's theory also begs the fundamental question of how, if not by means of flagella, bacteria move. Sometimes he appears to believe that they make lashing movements with their bodies, while at other times he argues as though they were capable of rotating in some unexplained manner, and thus screwing themselves through the water, without the assistance of any complementary reaction. This is obviously impossible, and propulsion by lashing, or by waves of contraction, is incapable of explaining the rapid motion of almost spherical cells in some species, notably the coccal swarm

cells of *Rhizobium*. The supposed bodily movements shown in Pijper's film appear to be no more than changes of aspect in a rotating, helical body. Thus Pijper's conclusions are quite unproven, and are, for the most part, unsupported by any kind of evidence, whereas a great deal of evidence has recently been accumulated in support of the classical view of the nature of flagella. The best of the recent papers upon the subject are those of Kingma Boltjes (58), and Houwink & van Iterson (52). The former summarises the evidence very clearly.

Especially valuable also are the observations of Ørskov (78) that the flagella of *Proteus* may be seen to sweep up and knock away particles of India ink, even when the body of the bacterium is stationary; those of Fleming, Voureka, Kramer & Hughes (42) and Fleming (41) upon the action of the flagella in penicillin-treated cultures of *Proteus*, much of which was actually confirmed in a film made recently by Pijper and exhibited before the Society for General Microbiology in London (April, 1950); the flagella can clearly be seen by phase-contrast microscopy, and propel the spirally coiled and globular bacteria found in these cultures in a manner quite incompatible with Pijper's theory; those of van Iterson (54) upon the mode of attachment of flagella, which may be seen, by electron microscopy, to pass through the cell wall; of Astbury & Weibull (1) upon the chemical structure of flagella, which are composed of a myosin-like protein; and of Bisset & Hale (23) upon the growth and development of flagella, showing them to protrude through the cell wall of germinating microcysts. Interesting electron micrographs have also been made by Conn & Elrod (32), Bartholomew (4), and many others. The existence and the nature of flagella was also discussed at a Symposium held by the Society of General Microbiology in April 1949, and much of the evidence upon both sides of the argument may be found in the published version, "The Nature of the Bacterial Surface" (111).

In a recent paper (89), Pijper examined the flagella of certain large spirilla. These were the organisms whose flagella were shown by van Iterson (54) to pass through the cell wall. Pijper claims that these are totally different from the flagella of *Salmonella typhi*, but as the main ground of this claim is that they are so obviously "classical" flagella, this conclusion does not follow logically, unless one commences with the premise that the flagella of *S. typhi* are not "classical."

Although his main thesis is unproven, Pijper's observations are not without interest. He has shown that many of the flagella of peritrichously flagellated bacteria may be twisted into a tail. This tail probably takes part in the propulsion of the cell, but this is not certain. When a tail is not formed motility is not impaired, which indicates that free flagella are at least equally efficient. This fact has been claimed by Pijper as evidence of the passive nature of flagella, but this reasoning assumes that flagella which cannot be resolved by dark-ground illumination do not exist.

It is interesting to recall that peritrichous flagellation is probably an adaptation to a terrestrial existence [Bisset (21)], so that it may not be

efficient under conditions of total immersion in a watery fluid, and may result in some degree of entanglement of the flagella.

Imsenecki's Artifact Theory

In view of the very considerable degree of agreement among microbiologists upon the nature of the bacterial nucleus, at least in very young cultures, which had been achieved by 1948, it is surprising to find that, in that year, the distinguished Russian microbiologist Imsenecki published a paper (53) in which he attempted to prove that the rod-like chromatinic bodies are artifacts, produced by the technique of staining with Giemsa. Subsequently, similar views were expressed by another Russian worker [Bondarenko-Zozulina (25)]. This conception is not, or was not, universal in Russia, as is shown by the interesting researches of Peshkoff (83, 84).

Imsenecki appears to be unaware that the nuclear bodies have been demonstrated by other techniques, in addition to acid-Giemsa, and he concentrates his attack upon the work of Robinow. Working with cultures as little as 20 min. old (in parenthesis, this is not, as he claims, a very young culture, but is as old as the inoculum from which it was made because many of the cells will not have commenced to grow) he attempts to show that a short period of staining produces the well-known "bipolar" effect; and a longer period causes the stained poles to migrate towards the centre of the cell, where they appear as chromatinic rods; after a still longer period of staining they coalesce at the centre of the cell.

It may be explained, in a few words, how this result is obtained. The inocula examined by Imsenecki presumably consisted of a mixture of vegetative cells, which had already germinated, or which had not achieved the microcyst stage before they were subcultured, and of cells at various stages in the process of formation of the resting nucleus [Bisset (15)]. Lightly stained cells, at any stage of growth, appear "bipolar," because only the surface layers of cytoplasm are stained; which, when seen across the thickest aspect, at the poles of the cell, give this appearance. This phenomenon, which is an optical illusion, is quite distinct from the staining of polar granules in other bacterial species, especially *C. diphtheriae*, which is discussed earlier. A more protracted period of staining may succeed in staining the nuclear bodies of young cells, and a still longer period, the central nuclei of resting cells. Thus the three stages described by Imsenecki do appear, after varying degrees of exposure to the staining solution; but not for the reason he suggests.

These structures can, in any case, be observed unstained by ultraviolet light [Piekarski (94)], by phase-contrast [Tulasne (112); Stempen (109); Pulvertaft (97)], and by dark-ground microscopy [Stoughton (110)]; they also can be demonstrated by a variety of staining methods. The various forms observed by Imsenecki do not occur simultaneously in young cultures, but only in old or germinating cultures such as, in fact, he used.

The L-Forms in the Bacterial Life-Cycle

It may now fairly be said that the once vexed controversy of the significance of the pleuropneumonia-organism-like L-forms, which are frequently found in cultures of bacteria, is a controversy no longer. Much work remains to be done in the elucidation of the exact nature of these structures and their importance in the bacterial life-cycle; but that they are derived from apparently normal bacteria, of many different genera, and may, even after prolonged culture in the L-phase, give rise in their turn to a new generation of bacteria, is not in dispute. The major point now at issue is the morphology of the units comprising the L-forms. The former problem has been discussed in the author's recent monograph (17).

Klieneberger-Nobel, once the protagonist of the view (62) that the L-forms, of which she is the discoverer, are parasites or symbionts of the bacteria, but recently (63) converted to the view of Dienes (34) that they are a phase in their life-cycle, nevertheless contends that the L-forms are much less bacterium-like than is suggested by Dienes (35, 36) and others. This extension of the argument hinges upon the problem of the minute structure of the L-forms, and this is exceedingly difficult to determine. There is little doubt that some L-elements closely resemble minute bacilli, both by light microscopy and also by electron microscopy [Smith, Mudd & Hillier (108)]. On the other hand, Klieneberger-Nobel (64) contends that the majority of particles are of dimensions comparable with those of viruses. It is also claimed that the production of L-forms from bacteria may be induced by various agencies, notably penicillin [Dienes, Weinberger & Madoff (37); Warbasse & Johnson (116)], and their serological and biochemical properties are the same as those of the parent strains [Weinberger, Madoff & Dienes (118)]. The problem is somewhat complicated by the contention of Freundt (44) that the L-forms, although admittedly part of the bacterial life-cycle, do not bear so close a resemblance to pleuropneumonia organisms as Dienes and Klieneberger-Nobel maintain.

The occurrence of small, bacillary forms, apparently reproductive in function, in cultures of bacteria, is quite well substantiated [Allen, Appleby & Wolf (2); Guélin (47); and others]. Such reproductive gonidia also occur in spirochaetes [Hampp, Scott & Wyckoff (49); see also Bisset (17)]. Whereas it cannot be asserted with confidence that these tiny bacilli are cytologically equivalent to the larger, parent forms, it equally cannot be disproved in the present state of our knowledge. It is only recently that the structure of comparatively large bacilli has been convincingly demonstrated, and no technique at present available seems likely to perform a similar service for these minute bodies. However, there seems little reason to assume a complex hypothesis where a simpler one will serve equally well to explain the observed facts.

The nature of the L-forms is bound up very closely with the problem of the nature of the pleuropneumonia organisms themselves, which is discussed in the final section.

The Cytology of Bacteria Infected with Bacteriophage

Several studies have been made upon the cytology of bacteria under phage attack. Electron micrographs have shown the disruption of the cells, but have given little information concerning the changes which precede this disruption. Studies of the distribution of the nucleic acids in the cell, in the early stages of phage infection, have been more illuminating.

There appears to be little doubt that different phages, even in the same strain of bacterium, produce different appearances in the infected cell; i.e., the lesion is characteristic of the infection, not of the host. This was indicated by the work of Luria & Palmer (72), and confirmed by Beumer & Quersin (7) and Quersin (98). Beumer & Quersin stated that, in the same strain of *Shigella*, infection with one strain of phage produced elongated cells with nuclei swollen into an amorphous mass almost filling the cell, whereas another strain of phage produced cells swollen in all dimensions, with figure-eight-shaped nuclei. Quersin (98) recorded various appearances of phage-infected nuclei, all commencing with an original swelling, and including amorphous masses, spherical bodies, and median rods. In *Pasteurella pestis*, P'an, Tchan & Pochon (79) observed an initial swelling of the nuclear bodies, which later degenerated into a central thread and finally disappeared. Upon the other hand, Murray, Gillen & Heagy (76), working with *E. coli*, found that the nuclear material was disrupted and distributed upon the walls of the cell. While there is little doubt, as has already been stated, that different lesions are produced by different strains of phage, the work of Murray and his collaborators is much more convincing in every way, and merits discussion in greater detail.

Two complementary staining methods were employed; the nuclear structures were stained positively by Giemsa and negatively by thionine, and the results obtained were mutually corroborative to a high degree. Shortly after the first contact with the phage, the nuclear material lost its characteristic form and was distributed in bands around the cell in approximately the original position of the chromosomes. Later, granules and concentrations of nucleic acids appeared in these bands, and it is believed that these may be foci of phage multiplication. These observations are, to some extent, confirmed by those of Boyd (26, 27) made by phase-contrast microscopy. A study by Luria & Human (71) confirms the general picture presented by Murray *et al.*, but also describes a strain of phage which does not appear to disrupt the nucleus of its host.

An entirely different picture of the effect of phage action on *Clostridium perfringens* is given by Guélin (47), who describes the appearance of coccoid elements in the culture, and subsequently of minute cocco-bacilli. He proceeds, however, to the conclusion that these forms may appear in ageing cultures, uninfected by phage, and that their appearance under its influence may be due to a nonspecific irritation. This phenomenon is probably more closely allied with those discussed in the previous section, and especially with the work of Dienes, Weinberger & Madoff (37), although these workers

claimed that phage did not elicit the production of L-forms in bacterial cultures in the same manner as did the action of other irritant agencies.

Exceedingly little is, as yet, known of the pathological appearances of phage infection in bacteria. Two facts, however, appear to be established. Firstly, that the appearance of the lesion is characteristic of the phage and not of the bacterium, which is in accordance with the conditions found in infections of other cells by other agents. Secondly, the nucleus is usually attacked and may provide material for the reproduction of the nucleoproteins of the phage.

The Relationships of Bacteria

Whereas eubacteria appear to form a close group, with many morphological and physiological characters in common, not only is their relationship with other natural groups, such as the algae and fungi, highly problematical, but there exist several groups of organisms which are usually classed with, or assumed to be related to bacteria, whose true affiliations are exceedingly dubious. Of these, one group, the ultra-microscopic viruses, ranges from such examples as vaccinia, which might be claimed to possess a number of characters (mainly negative) which are reminiscent of bacteria, to those forms, especially plant-pathogenic viruses, which appear quite unlike any other known, living creatures. Groups such as rickettsiae, spirochaetes, streptomyces, myxobacteria, blue-green algae, and pleuropneumonia-organisms, and isolated genera such as *Azotobacter* and *Caryophanon* are also difficult to classify in relation to bacteria. In some cases the problem has already been argued *in extenso* elsewhere; Pringsheim (90) has reviewed the relationship between bacteria and blue-green algae, and has concluded that they are not closely related. The systematic position of "trichobacteria," such as those described by Brunel (29) and Skuja (105), as well as by Pringsheim, which appear, superficially, to have affinities with both groups, is thus very obscure. Pringsheim considers *Beggiatoa*, usually described as a "sulphur bacterium," to be related to the *Oscillatoria;* a study of its morphology by Johnson & Baker (56) does little to suggest any close affinity with bacteria.

The author has shown (15, 17) that the type of life-history which is so obvious in myxobacteria is found, in a less elaborate form, in eubacteria, and has also suggested (21) that the former exemplify a high degree of specialisation to a terrestrial existence. The same applies to streptomyces, which resemble bacteria cytologically, but which have adopted a habit of growth which is adapted to the aerial distribution of the resting cell; a different type of adaptation to dry land from that evolved by myxobacteria, but exceedingly successful. Even the bacterial endospore is probably adapted primarily to aerial distribution; its proteins are concentrated for the sake of lightness, and it has thus acquired, at second hand, the remarkable resistance to heat and chemicals for which it is well known (21). The resemblance of myxobacteria and streptomyces to *Acrasieae* and moulds respectively is

almost certainly due to mere convergence, in the process of adaptation to a similar mode of life. It has been shown [Bisset & Moore (24); Morris (73)] that a series of forms, of different degrees of structural complexity, connect streptomyces with simpler bacteria, and that the microaerophilic, pathogenic *Actinomyces bovis* resembles eubacteria much more closely than streptomyces (73). The distinct differences between aerobic and anaerobic "actinomyces" were recognised by Rosebury (102), but many authorities continue to class them together [Waksman (114)], and even to place the entire group, thus synthesised, among the monilias [Bessey (6)]. *Streptomyces* are almost certainly bacteria and *Actinomyces* resemble eubacteria more closely than do, for instance, mycobacteria.

The problem of spirochaetes is rendered more than usually difficult because of the almost total absence of information upon their morphology. Electron micrographs by Babudieri (3) and Morton & Oskay (74), although interesting, are of little help in the elucidation of the problem. The observations of Hampp, Scott & Wyckoff (49) and of other workers (17) suggest that the gonidial method of reproduction, common in certain eubacteria (see section on the L-Forms) is even more frequently found in spirochaetes, whereas the evidence of DeLamater, Wiggall & Haanes (33) shows that single or multiple reproductive cysts may be formed. The dividing line between spirochaetes and spirilla, once believed to be well defined, is shown in a more dubious light by the observation that members of both groups may reproduce by this method and swim by means of polar flagella. The possession of a markedly spiral morphology appears to be an adaptation to an aquatic existence, and probably a primitive characteristic (21); for this reason it cannot be accorded any great taxonomic value. Polar flagella are probably also a primitive aquatic character and it is interesting to note that in a peritrichously flagellated water bacterium, described by Gray & Smith (46), the polar flagella are the most strongly developed.

Aquatic bacteria may, on the other hand, be sessile forms, either solitary or colonial, relying upon motile swarm cells for distribution. The two, main groups of sessile bacteria, the stalked caulobacteria and sheathed, filamentous chlamydobacteria, have been little studied. Klieneberger-Nobel (59) described the cytology of *Sphaerotilus natans*, a chlamydobacterium morphologically resembling the better-known iron bacterium *Sphaerotilus discophorus*, and from her observations the organism appears to differ very little from eubacteria. Caulobacteria were first described, as a group, by Henrici & Johnson (51). Since that time some observations upon *Caulobacter* have been published by Houwink & van Iterson (52), and an interesting "photoheterotroph" *Rhodomicrobium vannielli* has been more fully described by Duchow & Douglas (38) and Murray & Douglas (75). *Caulobacter* appears to have little to distinguish it from eubacteria, except that the terminal flagellum, with which the free daughter cell of a newly-divided sessile bacterium is provided (division being in the normal, transverse plane) is transformed into a stalk when the new bacterium attaches itself to the substrate.

Upon the other hand, *R. vannielli* is stalked but reproduces by the formation of a bud upon a relatively long filament. The physiology and cytological structure (but not behaviour) of the cell is bacterial, but it can only provisionally be ascribed to the caulobacteria. The nearest parallel to the mode of formation of the bud is the development of the microcyst in *A. bovis* (73).

The simple, unspecialised aquatic bacteria are almost certainly ancestral both to the specialised terrestrial bacteria and also to the more complex and specialised water bacteria. The relationship between the spiral bacteria, with polar flagella, from which the typical eubacteria are probably derived, and the flexible, apparently non-flagellated trichobacteria, from which, conceivably, the myxobacteria may have arisen, is highly problematical. The genus *Lineola*, described by Pringsheim & Robinow (92) and Pringsheim (91), consists of long filaments, resembling trichobacteria and similar in habitat, but motile by means of peritrichous flagella.

Several relatively large organisms, usually regarded as bacteria, have been examined cytologically. Of these, *Azotobacter* [Pochon, Tchan & Wang (96); Bisset (12); Eisenstark & McMahon (39); Eisenstark, McMahon & Eisenstark (40)] appears to resemble eubacteria in most respects, although exaggerating the yeast-like characters which are, nevertheless, found in many bacteria (see INTRODUCTION). Similarly, *Caryophanon* [Peshkoff (83, 84); Pringsheim & Robinow (92)], although large, resembles bacteria much more than the blue-green algae with which Peshkoff suggested that it forms a connecting link.

Luminous, halophilic organisms, examined by Johnson & Gray (57), although variable in form, had much in common with eubacteria.

A group of organisms whose relationship with bacteria has been the subject of much discussion, the *Rickettsiae*, has been investigated by means of the electron microscope by van Rooyen & Scott (103). These workers came to the conclusion that *Rickettsiae* resembled bacteria rather than viruses in their morphology.

The nature of the pleuropneumonia-organisms is closely bound up with the problem of the L-forms in the bacterial life-cycle, which has already been discussed. The general agreement which has now been reached upon the bacterial nature of the L-forms has its corollary in the almost inescapable conclusion of the bacterial nature of the pleuropneumonia-organisms, or at least in a considerable revision of the view, once widely held, that they belong to a distinct class of living creatures, or are, at best, distantly related either to *Actinomyces* or filterable viruses, upon the basis of their occasional production of branched filaments or of filterable, reproductive granules. This view is represented by the article of Sabin (104), who suggests a classification which places them in a class *Paramycetes*, and is also intended, apparently, to include almost any other forms which do not fit well into the present classification. Such taxonomic refuse-bins are greatly to be deplored, as also is the tendency to describe "missing links" (e.g., both *Rickettsiae* and pleuropneumonia-organisms as intermediate between bacteria and viruses). These attractive suggestions seldom withstand the test of time.

A general study by Dienes (34), based upon his own work and that of Klieneberger & Smiles (65), presents the conclusion that pleuropneumonia-organisms are definitely bacterial in nature, and that their peculiar morphology is paralleled by that of the reproductive L-forms of other bacteria. This conclusion is supported by the electron micrograph studies of Smith, Mudd & Hillier (107, 108).

This section is not complete without some discussion of the general definition of bacteria. Obviously the standard definitions have not stood up well to recent advances in knowledge. The author has suggested elsewhere that the title *Schizomycetes*, defined as fungi which reproduce solely by simple fission, is quite outmoded; the word has no advantages of euphony or priority over *Bacteria*, which should be readopted; the definition is even more completely falsified. In the present stage of microbiology when new, fundamental facts are constantly being added to the store of our information, it seems rash to attempt a hard-and-fast definition. The characters which now appear clearly established are:

Unicellular or multicellular organisms; reproduction by fission, simple or complex, or by gonidia; nucleus vesicular in resting cells, adopting a mitotic condition, semipermanently, in haploid vegetative cells; formation of haploid resting cells by a sexual or autogamous process, rapidly succeeded by reduction; rigid cell wall, usually slightly spiral in form; motility when this occurs, by spiral flagella.

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THE CYTOLOGY OF THE FUNGI[1]

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It was recently stated that in cytology, perhaps more than in any other science, progress depends upon manipulative skill and that type of low cunning which is needed to apply old methods to new uses. This truth is beautifully illustrated in the case of the fungi where, in cytological work, we deal with structures and artifacts lying close to the limits of visibility, and only the most cunning of cytologists succeeds in devising significant new methods. However, within the last two decades a sufficient number of ingenious new techniques have been conceived to further progress to the point where it can be said with some confidence that the general topography of the fungal cell has been comprehensively mapped. Nonetheless, our knowledge of the fine structure of the thallophyte body is still rudimentary, and we are decidedly ignorant of the formation and chemical nature of its organelles. Interest in the present is shifting from the descriptive to the experimental phases of cytology, and recent developments in quantitative optical microscopy and particularly in histo-chemistry insure that cytological investigation of the dynamic features of cellular development will become an increasingly important subdivision of biology.

In this review we have elected a phylogenetic approach to the subject, deliberately planned to accentuate the woeful lacunae in our cytologic information on the Eumycetes. No attempt is made to include all the researches of the period in question, and where reviews of particular segments of this field are already available, we have not hesitated to skirt those topics which seemed adequately covered.

Phycomycetes

In simplicity of form and life cycle, as well as by analogy with other thallophytes and protista, this group is commonly regarded as the most primitive of the fungi. They are usually coenocytic, multinucleate organisms reproducing asexually by the formation of motile or nonmotile spores born in thin or thick-walled sporangia, and sexually by the development of coenogametangia; these in turn produce either motile planonts or nonmotile coenogametes. The zygotes are commonly heavy-walled resistant structures which are capable of, or require, a long dormant period before germination. Many of the early researches on the development of sexuality in the primitive plants utilized these organisms, and these have been extensively reviewed by Sparrow (120), Whitehouse (136), and others.

Chytridiales.—Karling (60) recapitulated the cytological observations made upon this order before 1937, and he and his students have initiated

[1] This review covers the period from 1935 to 1950.

studies of nuclear behavior and cytomorphology in a number of species. This group employed the classic staining techniques, and their results show a surprising uniformity of nuclear behavior throughout the group. Sporangial mitoses in the chytrids are intranuclear and generally synchronous. The nuclei are relatively large, easily stained and contain a well-defined nucleolus. Their fixation and staining techniques do not sufficiently differentiate chromosomes so that reliable counts may be made, but haploid numbers from 4 to 12 are recorded. In most cases, the dividing nuclei show centrosomes or densely staining structures at the poles of the intranuclear figure, but Ajello (1) failed to find them in *Polychytrium aggregatum*. The large primary sporangial nucleus and the delicate sporangial wall of these forms suggest certain chytrid species as ideal material for studies on mitotic chromosome morphology, with some of the newer smear techniques, although Antikajian (2) implies that these techniques were unsuccessful in *Asterophlyctis sarcoptiodes* because of the presence within the sporangium of much refractive lipoidal material. Such refractile bodies have been widely reported in this order, but beyond the fact that they are primarily lipoidal and contain acetone-insoluble fractions, their composition is poorly understood.

The function and structure of the curious nuclear cap, which is characteristic of the motile planonts in the monoflagellate series of aquatic Phycomycetes, has occasioned great debate among chytridiologists. In *Cladochytrium replicatum*, Karling (60) believes the nuclear cap arises from the fusion of numerous basophilic granules which appear in the cytoplasm during sporangial cleavage. These granules may be derived from fragmented nucleoli or they may represent chondriosomes; and the Feulgen reaction does not clearly differentiate the nuclear cap from the nucleus. In *Endochytrium operculatum*, Hillegas (48) found the cap staining strongly with basic dyes and giving a weak Feulgen reaction. Vital staining did not support the theory of chondriosomal origin. The observations of Roberts (102) tend to confirm Karling's idea that the cap is synthesized from lipoidal granules, and he considers that it functions as a reserve food body. In *A. sarcoptiodes* (2) it is weakly Feulgen-positive, in *P. aggregatum* (1) it is Feulgen-negative and stains like the nucleolus. It can, however, be stained in the living condition by Janus green which is rapidly reduced to its pink leuco-base. It is obvious that more precise studies are required to still this controversy.

The thallus walls here give chitosan reactions (1, 2, 102), indicating the presence of fungal chitin, and may also contain cellulose (48, 102), and an unidentified substance which is neither chitin or cellulose (102). The sporangial operculum of species of *Nowakowskiella* (102) and the zoned sporangial hull of *Rhizophydium coronum* (46) are composed of pectic substances.

The sexual nature of the thick-walled resistant sporangia developed by some chytrids had been tacitly assumed by many early workers. None of the modern workers except Shanor (114) has found any cytological evidence indicating either karyogamy or meiosis during the formation or germination of these structures. A nuclear fusion may occur during the development of the

resting spore of *Nowakowskiella hemisphaerospora*. In all other reported instances the resting structure is an apogamous zoosporangium which, in response to environmental conditions, develops a specialized wall.

Blastocladiales.—The recent review of this group by Emerson (39) makes further discussion of nuclear behavior and life cycles here superfluous, although it should be emphasized that the demonstration by Emerson & Wilson (38, 40) of meiotic divisions in the resistant sporangia of species of *Allomyces* is the first satisfactory cytological account of this process in any aquatic Phycomycete. The characteristic nuclear cap and peculiar "side body" of the motile planonts of this order have been the source of much interest since Hatch (47) postulated in 1935 that the nuclear cap arose through chondriosomal aggregation. Hatch's thesis was developed principally upon the basis that the cap was Feulgen negative, and, because of the disparity in size between caps in oppositely sexed gametes, he felt that it might function in sex determination. Guilliermond (44) was disposed to question Hatch's theories, and the inconclusive results of the research on the nuclear cap in the chytridiales have added little to support Hatch. In a very careful study of the zoospores of three species of *Allomyces*, Ritchie (100) demonstrated Feulgen-positive granules in the nuclear caps. He points out quite logically that the nuclear cap is acid in reaction and stains with basic dyes, whereas mitochondria, properly fixed, are basic and become chromatic in the presence of acid dyes. He concludes that the cap is partly nuclear and partly cytoplasmic in structure, but can find no evidence for its mitochondrial nature. While we realize that the specificity of the Feulgen reaction is open to some question, Ritchie's observations seem sufficiently critical so that there appears no reason for further serious consideration of Hatch's conclusions. The answer to this puzzling and phylogenetically important question may reside in the relative concentration of different types of nucleic acid in the cap, and an examination of the effect of ribonuclease upon the cap might be most instructive. We still know nothing of the structure of the "side body" which is unique in the motile spores of this order, although reports (23, 125) of its staining reactions indicate that it may be composed partly of nuclear elements.

Lagenidiales.—The only modern cytological study of a simple holocarpic biflagellate Phycomycete, that of McLarty (78) on *Olpidiopsis achlyae*, brings out several interesting comparisons between the cellular organization in these fungi and the morphologically analogous chytrids. Nuclear divisions here are mitotic and simultaneous, but the great enlargement of the primary sporangial nucleus is not noted. Clearly demarcated centrosomes are present at the poles of the intranuclear spindle. In sharp contrast to that of the Chytridiales, the cell wall in *Olpidiopsis* gives a strong cellulose reaction and the zoospores lack a nuclear cap. McLarty has suggested that the nucleolus serves as a storehouse for reserve chromatin, but this is hardly borne out by the different staining reaction he observes between nucleolus and chromatic reticulum. Frequent fusions are observed between thalli of unequal sizes, but

the sexual nature of these fusions has not been confirmed. While plasmogamy undoubtedly occurs, McLarty has found no indication of either karyogamy or meiosis. Some early workers were inclined to regard thallus anastomosis involving plasmogamy as a primitive type of sexuality, but until karyogamy can be demonstrated the evidence for a sexual cycle here is very tenuous. The cytological organization of *Olpidiopsis* confirms the current opinion that the affinities of this order are with the biflagellate series of the Phycomycetes, and that the overall morphological similarity with the Chytridiales merely represents a type of convergent evolution within a similar biosphere.

Saprolegniales.—The broad thin-walled hyphae of species of *Saprolegnia* have lent themselves admirably to the study of cytoplasmic structure, and Guilliermond (44) has extensively reviewed the many contributions that he and his colleagues have made to a knowledge of this subject. In *Saprolegnia* the chondriome is composed of granular mitochondria aggregating at meristematic regions and elongated sinuous chondriosomes located in areas of diminished cytoplasmic activity. The interrelationships of these Feulgen-negative lipoprotein bodies, and their integration with the vacuome and the other ergastic inclusions have been intensively investigated by the French school. The method of choice has been the use of selective vital dyes, coupled with fixation procedures utilizing strong oxidizing agents which presumably convert the unsaturated fatty acids of the chondriome to hydroxylic acids and thus render them chromatic with acid dyes.

Most modern investigators of life-cycles in this group are in agreement on the occurrence of fertilization in the oogonium, although minor differences in the nuclear behavior of different species are reported (138). Meiosis is assumed to occur in either the "dormant" or the "germinating" oospore, but the process has not yet been demonstrated in any species. Haploid chromosome numbers from 1 to 16 have been reported in different species, but the lack of agreement on basic numbers and the generally minute and stylized diagrams of mitotic figures leads the impartial observer to the conclusion that many of these counts may be subject to later revision.

The coenocentrum, a peculiar inclusion prominently mentioned in the literature, presents several puzzling features. A dark staining area in the oosphere, it appears to be concerned with the process of karyogamy, but it is not known whether it represents dispersed products of the gametic nuclei, coacervated elements from the oosphere cytoplasm, or merely a monument to inadequate fixation. Shanor (113), having demonstrated the presence of astral rays associated with the nuclei of young zoospores, has further speculated about the role of centrosomes and astral radiations in delimiting oospheres and zoospores. The general occurrence and significance of these centrosomes and rays requires further clarification, since there are many discrepancies in the reports concerning them (54, 93).

Leptomitales.—Kevorkian's (61) study of *Apodachlya* and *Sapromyces* has clarified the systematic position of these fungi. The nuclear behavior in the reproductive structures of *Apodachlya* indicates saprolegniaceous affini-

ties, whereas *Sapromyces* shows somewhat closer ties with a number of species in the Peronosporales.

Peronosporales.—The very extensive cytological comparison of several species of *Pythium* by Saksena (104) emphasizes, on the basis of nuclear behavior and extranuclear structure, the intermediate position of the Pythiaceae between the Saprolegniales and Peronosporales. Saksena's comments concerning the coenocentrum are particularly enlightening. Mundkur (79), investigating oogenesis in *Phytophthora himalayensis*, a form with amphygynous antheridia, notes several points of difference between his material and several species with paragynous antheridia, which cast some light upon the evolution of amphigyny. Blackwell's (7) succinct account of nuclear behavior in the paragynous *Phytophthora cactorum* offers an interesting comparison with the foregoing study. Neither worker noted a coenocentrum, but Blackwell points out the very significant fact that the favored female nucleus in the oosphere is in association with an accumulation of unsaturated fat which is strongly reactive to osmic acid. This single observation may go far towards explaining the mystery of the coenocentrum. Thomas' study (129) of the cell wall structure of species of *Pythium* indicates that a basic skeleton of chitin is overlaid with cellulose impregnated with fatty acids. There is an outer sheath of callose surrounding this complex.

McDonough (74) has completely summarized the literature to 1937 on nuclear behavior in the Peronosporaceae, and has carried out the most detailed and convincing study of nuclear behavior and cytoplasmic structure in this family which has yet appeared. His observations upon the fate of the nucleolus of *Sclerospora graminicola* during meiosis are quite unusual. During the first division of the fusion nucleus the nucleolus, which is relatively very large, elongates during early telophase into a dumbbell-like structure, the swollen ends of which lie within the reticula of the telophase nuclei. As the nucleolar division is completed by the pinching-off of the daughter nucleoli, the nuclei remain connected by a slender thread which presumably represents the remains of the prophase membrane. Neither centrosomes nor astral rays were observed in association with any of the nuclei in the oogonium. Following the two meiotic divisions of the fusion nucleus, the oospore germinates. McDonough feels the haploid chromosome number is probably 14. In *Cystopus evolvuli* (128), the haploid number is reported to be 16. In both species a coenocentrum is found, and when the developing oospore is fixed during fertilization with strong oxidizing reagents and stained with acid dyes, the coenocentrum is still demonstrable. The arrangement of mitochondria (75) in the ripening oogonium suggests a possible connection between these organelles and the developing oospore wall. Extensive histochemical tests of the "oil body" in the mature oospore have failed to establish anything more definite than that it is not soluble in fat solvents, and that there are indications that it may be in the nature of a carbohydrate-protein complex. Recently McDonough (76) has utilized the nuclear behavior of *Sclerospora macrospora* as a basis for a discussion of its taxonomic position,

and points out that there may be a valid cytological basis (77) for separating the family Albuginaceae from the other families of the Peronosporales. Bosc (8), comparing mitosis and meiosis in *Plasmopara vitacola*, has emphasized the point so frequently alluded to by fungal cytologists that the haploid nuclear divisions seem to be "promitotic" or "amitotic" whereas the diploid divisions are normally mitotic. This appearance might readily result from the astringent effect of various popular fixation and dehydration techniques, accentuated by size differences in the nuclei involved.

Mucorales.—For the past 50 years the question of nuclear behavior within the coenozygote of the Mucorales has been a source of controversy. The basis for these conflicting theories became evident when Cutter (24, 25) completed a comparative study of the complete life cycle of 15 species. Four general types of nuclear behavior were recognized, and most of the discrepancies of the earlier accounts could be reconciled in terms of these patterns. The interpretation of these life-cycles requires an appreciation of the changes in appearance of nuclei in response to metabolic conditions. In areas of active metabolism and cyclosis the nuclei are expanded and, in both haploid and diploid phases, show a well-defined nucleolus and reticulum. Regions of reduced cytoplasmic activity, on the contrary, contain nuclei which are unexpanded and in which the chromatic elements are clumped. Intermediate stages will be frequently encountered, and the unexpanded condition does not necessarily indicate loss of function or senility, since unexpanded nuclei may expand if they enter areas of increased or renewed metabolic activity. These stages may be readily demonstrated in adequately fixed and stained material by comparing the nuclei of young and old mycelia or of dormant and germinating spores. This concept is not peculiar to the Mucorales and is probably equally applicable to other fungi, as DeLamater (29) and Savile (110) have shown. Cutter's investigation of the chondriome in the Mucorales revealed a condition very similar to that found elsewhere in the Phycomycetes. He was of the opinion that the central globules or elaioplasts result from mitochondrial aggregation and degeneration, since some unpublished observations indicated that they may be composed of phospholipid. Tarwidowa (124) arrived at a similar conclusion about the fat globules of the related entomophthoraceous *Basidiobolus ranarum*. Sjowall (117, 118) confirmed Cutter's findings in regard to nuclear behavior patterns in *Mucor hiemalis* and in those stages of *Phycomyces blakesleeanus* which he investigated. In *Rhizopus nigricans* he finds only one surviving diploid nucleus undergoing meiosis instead of several, as reported by Cutter. Callen (13) had earlier reported a nuclear behavior in *Rhizopus sexualis* somewhat intermediate between the *Rhizopus* and *Phycomyces* patterns outlined by Cutter, but he was unable to germinate the zygospores and could not complete the study.

A unique aspect of fungal cytology is illustrated by the extensive studies of Castle (15 to 18) on the fine structure of the cell wall of *Phycomyces* and its relation to the general problem of spiral growth. Castle demonstrated

that, during elongation, the sporangiophore shows spiral growth sinistrally oriented, during sporangium formation growth is temporarily unoriented, and, after development of the sporangium is complete, growth is renewed in a dextral direction but shortly reverses again sinistrally. Preston (89) has likened this behavior to the extension of an axially loaded flat helix. In spite of extensive investigation, the explanation of these phenomena is still obscure.

Johannes (58) recently discovered that, by carefully controlling the pH of his cultures and utilizing vital dyes, it is possible to stain the plus and minus gametangia and suspensors of *Phycomyces* differentially. Similar differential staining is shown by the oogonia and antheridia of *Saprolegnia*. If this phenomenon can be generally demonstrated in the lower fungi, it will be the first reliable method of visualizing a chemical difference between the sexes in isogamous organisms. He has also (59) found a slight, and possibly significant difference in the number of nuclei in the multinucleate plus and minus sporangiospores of *Phycomyces*, and this difference is apparently consistent in spores from primary and secondary sporangiophores.

Entomophthorales.—The cytology of these insect parasites has received scant attention in recent years. Couch (22) discussed some phases of nuclear behavior in *Conidiobolus Brefeldianus* and investigated the curious vacuole of the conidiophore, which plays a role in maintaining the turgor of this structure and in the forceful discharge of the conidium. The staining reactions of this vacuole are quite different from those of ordinary hyphal vacuoles, and if it is plasmolyzed the conidium is not discharged. He also studied the cell walls of several members of this group, finding cellulose present in *Basidiobolus*, and lacking in *Conidiobolus*, *Ancylistes*, and *Empusa*. Schweizer (112) succeeded in cultivating a number of species of *Empusa* on cold-sterilized media and used this material for a painstaking study of the cytology of *Empusa muscae* throughout its life history. With the aid of a new staining technique (111), he obtained some magnificent nuclear preparations. The student of comparative cytology in the fungi, after glancing at his photographs, will be impressed by the large size and chromaticity of these nuclei compared with the closely related Mucorales. It becomes apparent, as will be seen later, that large nuclear volume may be a common characteristic of obligate fungal parasites of both plants and animals.

Ascomycetes

The recent critical essay on the life cycle and sexuality of the Ascomycetes by Martens (72) has greatly simplified the task of the present reviewer. This monumental survey has covered the vast and complicated problem so thoroughly and in so critical and unbiased a fashion that a further review of this field would be entirely superfluous. Consequently, in our discussion of ascomycete cytology, we will concentrate upon certain aspects not covered by Martens and upon contributions appearing since his publication and will make no attempt to review material included in his treatment.

Interest in ascomycete genetics has resulted in the appearance of many papers which contain observations, not in themselves cytological, but contributing greatly to our understanding of cytological behavior. Since not all these contributions could be listed here, we have sampled them with an eye to illuminating the possibilities in a genetic approach to fungal cytology.

Endomycetales.—The structure of the yeast nucleus has been a controversial topic for almost 75 years, and numerous publications purporting to define the yeast nucleus have appeared. The early work on the problem was summarized by Wager (130), who himself really laid the cornerstone of the present disagreement. According to him, the nucleus or chromatic body described by earlier workers was actually the nucleolus, the large central vacuole of the yeast cell represented the nuclear vacuole, and the metachromatic ergastic material which might be present in the vacuole was the nuclear reticulum. Wager's memoir provoked such a swarm of contradictory opinions that in 1910 he and Peniston (131) reexamined the problem in great detail and bolstered their previous opinions. They have reconciled the divergent viewpoints of their opponents as follows: "The conflicting accounts . . . are probably not due entirely to errors of observation but to remarkable variability in the behavior of the nucleolus to stains and reagents, to the variations in the nuclear vacuole and its chromatin contents and to the varying amounts of volutin, glycogen and phosphorus found in yeast at varying stages of development." At this juncture we will digress to point out that this same devious and unpredictable behavior on the part of the yeast plant and its nucleus has been invoked by all the exponents of unusual nuclear behavior in *Saccharomyces,* and not at all by those investigators who have favored more orthodox interpretations of nuclear structure.

Guilliermond (43) reviewed the work of the intermediate period 1910 to 1936 and demonstrated rather convincingly that a number of yeasts in addition to *S. cerevisiae* possess a characteristic chromatic body outside the vacuole which stains with basic dyes like any fungal nucleus, whereas the so-called nuclear apparatus of Wager & Peniston (131) was fixed by the oxidizing agents which preserve cytoplasmic structures and was stained by acid dyes. Richards (98) attempted to demonstrate mitosis in yeast by the use of colchicine, but did not obtain definitive results. Renaud (95) ascertained the presence of centrioles at the poles of the chromatic body during its division, and Levan (65) and Sinoto & Yuasa (116) described a number of chromosomes during the division of this chromatic body. Badian (4) was the first to indicate that the chromatic body was Feulgen-positive, but in other respects his elaborate account of the divisions of this body are not convincing and, in Guilliermond's words, "His illustrations are altogether too diagrammatic, and fit neither previous accounts nor his own microphotographs." Caspersson & Brandt (14), studying the quantitative absorption of ultraviolet radiation by different regions of the yeast cell, found cytoplasmic granules containing ribose nucleoprotein, and suggested that these might be the equivalent of the nucleus. However, their terminology is such that it is difficult to

determine what structures they had in mind, and the account lacks conviction. Jeener & Brachet (57) indicated that yeast may be peculiar among fungi in possessing a high ratio of ribose to desoxyribosenucleic acid, and Dufrenoy (36) has discussed the cytological implications of this aspect of the problem. It is easily understood that a high cytoplasmic content of ribose nucleic acid would create difficulties in the staining of the yeast cell by classic techniques, and subsequent interpretation of structure would be a very precarious proposition. In 1945, Lindegren (66) rekindled the smouldering controversy by resurrecting Wager & Peniston's interpretation and embellishing it anew. Since then, in a number of diverse (68, 69) publications, he has advanced the hypothesis that the chromatic body is only a centrosome, the cytoplasmic vacuole is in reality the nucleus and contains a Feulgen-negative nucleolus, and that the Feulgen-positive "chromosomes" contained within this vacuolar "nucleus" are composed largely of volutin (67). He has marshalled an incredible array of biochemical minutiae in support of this position, and in his recent voluminous compilation (68) on the structure of the yeast cell, he has dismissed or ignored the case developed by most of his critics on the basis of their inadequate cytological techniques. It is somewhat difficult for a conventional cytologist to achieve a dispassionate appraisal of Lindegren's work, for his techniques are so inappropriate and his interpretations so sweeping that they can hardly be evaluated in terms of earlier research. A perusal of the numerous photomicrographs he presents will perhaps reveal better than words the inconsistencies in his cytological observations. Lindegren has leaned heavily upon Rafalko's (90) demonstration of Feulgen-positive threads in the vacuole, but it must be recalled that aldehydes and pentoses, as well as both types of nucleic acid, have been known to give the Schiff reaction after sufficient hydrolysis, so that this argument is not incontrovertible. He has also drawn evidence from Wiame's (137) experiments on the deposition of volutin in phosphorus-starved yeast cells to bolster his hypothesis that these "chromosomes" are coated with volutin. Ranganathan & Subramaniam (91, 92, 122, 123) have criticized Lindegren's analysis of the nuclear apparatus and have themselves confirmed and expanded the traditional concept. Nagel (80), in an extensive reexamination of the question using strains supplied by Lindegren, coins the terms "magnicorp" for the vacuole, and "parvicorp" for the chromatic body. Her graphic presentation of the variable number of granules per magnicorp in different cells poses a question concerning Lindegren's interpretation of these bodies as chromosomes. If her counts are correct, and these really were chromosomes, one might be tempted to speculate that the explanation of the curious genetic ratios obtained by Lindegren in some of his yeast crosses (69), and previously attributed to the activity of a cytogene, may be found in the irregular segregation of these "chromosomes" during mitosis. Recently, DeLamater (30, 31), using a new technique (28), presented a cogent analysis and a magnificent set of photographs of mitosis in *S. cerevisiae* which leave little doubt concerning the definition of the nucleus and its normal behavior in mitosis.

In connection with the reported variation in the chromosome number in strains of *Saccharomyces*, Subramaniam (121) has detected evidences of polyploidy in some of his strains, although genetic confirmation of this is still lacking. The recent localization by Nickerson, Krugelis & Andresen (81) of a pyrophosphatase and a phosphomonoesterase on or in the immediate vicinity of the chromatic body, further substantiates the claim that this structure has a metabolic function akin to the traditional nucleus.

A feature throwing some light on the problem of the yeast nucleus is a comparison of the nuclear structure in the various heterokaryotic members of the *Endomycetales*. Nagel's (80) discovery of many "parvicorps" in some wild yeasts, the dikaryon shown by Renaud (94) in a species of *Saccharomyces*, and the multinucleate condition so strikingly revealed in *Blastomyces* by DeLamater (29), all indicate that nuclear behavior in this group is very comparable to that in other filamentous fungi. A possible explanation of the variability in staining reaction among yeasts may be found in the investigation of Di Carlo, Schultz & Fisher (32), who find great variation in nucleic acid content of yeasts under various conditions of culture. In the present controversy concerning the definition of the yeast nucleus, further argument over terminology seems futile. The critical cytologist will hardly be impressed with Lindegren's (66) summation to the effect that "chromosomes are structures which perform a specific biological function rather than specific chemical compounds." The bulk of evidence indicating the presence of a nucleus of the conventional fungal type, dividing in the normal manner, is overwhelming.

Gymnoascales.—Cole (20) briefly studied the ultraviolet absorption spectra of various regions in the spores of *Trichophyton mentagrophytes*. His results indicate that this method still lacks precision on such small objects, but that greater refinements may make it a most useful diagnostic method for cellular constituents. Baker (6) investigated *Penicillium notatum* and established a cytological basis for the heterokaryotic behavior demonstrated in this species by Pontecorvo (87, 88).

Pezizales.—Further contributions to the long controversy over the occurrence of double fertilization and brachymeiosis in Ascomycetes were made when Olive (85) and Hirsch (51) respectively reexamined *Patella melaloma* and *Pyronema confluens* with the aid of the aceto-carmine smear technique (26). Neither author was able to confirm earlier reports of brachymeiosis in these forms, and the results obtained by this newer technique are sufficiently clear to remove any lingering doubt that may still persist concerning the occurrence of these phenomena.

Hypocreales.—The recent exposition by Hirsch (49, 50) of the chromosomal basis of sexuality in *Hypomyces solani f. cucurbitae* is one of the more significant recent contributions. Here hermaphroditic, male, female, neuter, and homothallic strains have been isolated (45). At diakinesis, in the asci of perithecia produced in crosses involving all combinations of these strains, the following chromosomal complements are found. Hermaphroditic by

hermaphroditic yields four bivalents, thus indicating the hermaphroditic thalli have four chromosomes. These are morphologically distinct: #1 is long, #2 and #3 are intermediate, and #4 is very short. Hermaphroditic by male gives three bivalents and one univalent, apparently chromosome #2. Therefore male thalli possess only three chromosomes. Hermaphroditic by female likewise yields three bivalents and one univalent, in this case chromosome #3. Thus female thalli also contain only three chromosomes, but the opposite sex chromosome is included. This most unusual situation is further confirmed by the results of the cross male by female, which gives two bivalents and two univalents. As a result of frequent nondisjunction of the sex chromosomes during division in this cross, neuter races with two chromosomes and hermaphroditic races with four chromosomes are frequently obtained. All the homothallic races studied in this organism were found to have six chromosomes. Hirsch suggests that this may represent a kind of amphidiploidy as the result of chromosomal doubling following the cross of male by female. More recently, she (52) and her colleagues have surveyed chromosome numbers in the species of *Gibberella*, *Calonectria*, and *Nectria*, which have *Fusarium* as their imperfect stage. She finds six chromosomes in *Gibberella* and seven in *Calonectria* and *Nectria*. As has been pointed out by Cutter (27), chromosomal analysis of this type may be of considerable help in unraveling relationships in the larger fungal groups, and Hirsch's observations should stimulate further cytological examination of sexually aberrant races.

Sphaeriales.—The cytogenetics of the fungi received a tremendous impetus in 1945 from McClintock's (73) detailed study of the chromosomal morphology of *Neurospora crassa*, and her demonstration of the applicability of the smear technique to ascomycete chromosomes. Lindegren & Rumann (70) had earlier experimented with a smear method on *Neurospora*, with less definitive results. Singleton (115) has developed some modifications of the method, involving the use of cytase to soften the ascus wall prior to smearing. McClintock's study reveals a number of points of great theoretical interest to the fungal cytologist, not the least of which is the fact that the genetic constitution of a fungus may greatly influence its staining reactions. She has described an ingenious technique for determining spore order in the ascus, and has pointed out that synapsis in *Neurospora* differs from that encountered in most organisms in that the synaptic attraction occurs between partially condensed rather than fully extended chromonemata. Whether *Neurospora* is unique among fungi in this respect is not known, but the question deserves careful consideration. McClintock found seven chromosomes in *N. crassa* and *N. tetrasperma*, and Fincham (41) has recently surveyed a number of other strains of these species, finding seven to be the haploid number in all of them. He asserts that if six chromosome strains do occur, as has been reported (27), they would be expected to be rather sterile when crossed with seven chromosome lines. This need not necessarily be the case if we consider the analogous situation shown by Hirsch in crosses in-

volving unequal chromosomal complements in *Hypomyces*. A study of the chromosomal behavior in the ascus from crosses between strains of *Glomerella cingulata* by Lucas (71) has not revealed a cytological basis for the curious segregation of mating types which is known to occur. Dowding & Buller (34) have developed an ingenious technique for determining the rate of nuclear migration through mycelia and have measured nuclear movement in heterokaryons of *Gelasinospora*. There has been some speculation over the data of Zickler (140) on *Bombardia lunata* and Wulker (139) on *Neurospora sitophila*, which seemed to indicate that both first and second division chromosomal segregation occur in these genera. Dodge (33) has suggested that a more acceptable explanation might involve first division disjunction followed by chromatid crossing-over. A careful reanalysis of these data by Ryan (103) has shown that there is no acceptable basis for the earlier interpretation, and that for the present we must consider that in the molds, as elsewhere, homologous kinetochores are segregated in the first division and divide in the second division. Catcheside (19), after a statistical reanalysis of the data on segregation for ascospore color in *Bombardia*, points out the interesting and as yet unexplained fact that there is a biased segregation of mutant genes from their wild-type alleles. In this case the dark-colored spores from the wild-type allele tend to be segregated to the upper spindle poles during meiosis, while the mutant, light-colored spores are segregated to the lower poles. Since this biased segregation occurs within the ascus, the bias must depend upon either regional differentiation in a single ascus or upon a difference of behavior between the biased chromosomes. Further cytological investigation of this interesting organism would be of great interest.

Basidiomycetes

In this group the reproductive apparatus, the basidium, is considered to be homologous with the ascus but differs in that the spores are born exogenously and the spore number is usually reduced to four or two. Greis (42) has worked out the homology of the clamp connections in this group with the ascomycetous crozier and presents the cytology of a number of intergrading stages which make it appear probable that a direct evolutionary line may be established between these groups.

Ustilaginales.—The very small unexpanded nuclei and the thick walls of the chlamydospore have impeded cytologic analysis of nuclear division in the smuts and no study using thoroughly modern staining techniques has yet appeared. It has been well established by genetic methods that the uninucleate plant is haploid and that infection of the host is normally accomplished by the dikaryonic or diploid mycelium, and by inference the chlamydospore is commonly regarded as diploid. However, Sampson's (106) pithy comment, "It would be unwise to become dazzled by the fascinations of smut genetics, and to forget how few are the established facts concerning chromosome behavior in this group" is still pertinent today in spite of the appearance of several intensive investigations into nuclear behavior in various species. Sampson has summarized the known life cycles in the smuts

up to 1939. Two species are known which will complete their life cycle in culture without the presence of the host plant. In *Ustilago crameri*, Wang (133) finds no sporidia produced and the promycelium develops directly into vegetative mycelium in which the dikaryophase is initiated by hyphal anastomoses. She figures nuclear divisions involving four chromatic bodies in the promycelia and believes the haploid chromosome number to be two. Leach & Ryan (64) making a similar study of *Ustilago striiformis f. poae-pratensis* think a true diploid mycelium is formed. Their assumption is based on nuclear volume and appearance, and they also find a haploid chromosome number of two. Hirschorn (53), in a comparative investigation of a number of species observes a surprisingly uniform nuclear behavior in all of them. She finds the chlamydospore is diploid, meiosis usually occurs in the first two divisions of the diploid nucleus but may be delayed until the third or later divisions, and the haploid chromosome number is, in all cases, two. In addition to nuclei, she finds many chromatic bodies of undetermined function in the promycelia and mycelia. It is obvious from her account that the cytological methods she used are not well suited to this work, and it must be admitted that the chromosome counts proposed by all these workers are based upon very tenuous evidence. In fact, it is questionable whether chromosomes have actually been demonstrated at all. Recently Perkins (86) has proposed a method of tetrad analysis based upon his study of *Ustilago zeae* which makes possible the mass analysis of nonlinear tetrads. By simplifying genetic analysis of these organisms, this technique should materially contribute to the solution of many puzzling problems in nuclear behavior in the Ustilaginales.

Uredinales.—In some respects the most significant recent contribution to an understanding of nuclear behavior in the fungi is Savile's (110) comparative account of several rust species. Using a number of techniques not previously utilized on the fungi, he was the first to point out clearly that nuclei, irrespective of their chromosome complements, expand and contract in various portions of the mycelium and spores in relation to the physiological state of the cytoplasm. Another fact which he has pointed out that may be of great importance in arriving at an understanding of the physiology of obligate parasitism, is the staining reaction of the rust nucleus. With certain of his techniques the nucleus of the rust stains like the host plant nucleolus, while the rust cytoplasm stains like the host nucleus. Savile's concept of the rust nucleolus is somewhat unorthodox and in the light of data presented by Olive, does not seem a logical interpretation. Olive's (84) recent study of *Coleosporium vernoniae* contains the most complete account of meiosis and chromosome morphology in a rust which has yet appeared. His remarks and photographs indicate that these organisms are very favorable cytologic material and a cyto-taxonomic study of speciation in them might be most rewarding. Olive (83) has also investigated spermatium formation in *Gymnosporangium clavipes* and his account emphasizes the cytological and functional similarity of these structures with the microconidia of the Ascomycetes.

Thirumalachar's investigation of two unusual Indian rusts brings out the fact that the production of particular spore types in this group is more apt to be dependent upon physiological conditions in the host and environment than upon the nuclear number or chromosome complement of the mycelium at a particular stage. In *Uromyces aloes* (127), the terminal cell of the promycelium is bi- or tri-nucleate and fertile, while the basal cell is uninucleate and sterile. Basidiospores are not developed and the terminal cell of the promycelium develops an infection hypha which penetrates the host directly. *Cytospora oleae* (126) produces binucleate basidiospores and bears its spermogonia on binucleate mycelia. The spermatia are uninucleate but aeciospores are frequently observed within the spermagonia. When aeciospores are sown upon very young leaves they give rise to mycelia bearing spermagonia, but sown upon old leaves the resulting mycelia develops telia. In *Endophyllum sempervivi*, Ashworth (3) has demonstrated that while the spermatia may be functional they are not essential to the diploidization process. The origin of the dikaryophase and the role of spermatia in establishing it has been reviewed at length by Wang & Martens (134).

The extensive literature upon the cytology of host-parasite relation has been summarized in two reviews by Rice (96, 97). A recent study which is particularly pertinent to this problem is that of Humphrey & Dufrenoy (55) on *Puccinia coronata*. Their comparison of infected and uninfected tissues reveals that infected tissues secrete externally certain phosphorus compounds which they would normally retain. There is also internal vacuolar secretion of substances, probably pyridoxin, which are capable of coacervating phenolic compounds. This coacervation may in turn be correlated with a dispersion of nucleotides and possibly with the resulting decompensation of respiration. The balance between these factors determines whether the rust behaves as pathogen or parasite and hence whether it will survive or not. These observations are of particular interest when compared with Savile's remarks upon the staining reaction of rust mycelium and nuclei and lead to the hope that a clue to the true nature of the host-parasite relationship may be found in histochemical investigations.

Auriculariales.—In the primitive rust-like organism *Herpobasidium filicinum*, Jackson (56) finds a most peculiar delayed meiotic division in the cylindrical basidium. The first division which is presumed to be reductional occurs within the basidium and the nuclei migrate into the spores. The second equational division which would ordinarily complete the meiotic process is apparently delayed until the germination of the basidiospores. Although Jackson's report is very convincing, this situation, which is unique among fungi, requires further confirmation. Baker (5) presents an interesting account of the relation of nuclear division and spindle orientation to basidial structure in *Helicogloea*.

Tremellales.—Wheldon's (135) extensive study of cytologic detail in the basidia and basidiospores of a number of genera in this order has provided some data on interrelationships within the group.

Agaricales.—The known chromosome numbers in the *Agaricaceae* have been summarized by Wakayama (132) and Buller (12) has discussed in

great detail many of the aspects of diploidization in these fungi, and its significance in terms of fungal evolution. Brodie (10) finds that oidia or microconidia are almost universally present in the fleshy hymenomycetes and are generally uninucleate. They are born on both haploid and dikaryotic mycelium and apparently function primarily in diploidization although they may serve as reproductive conidia. The domesticated mushroom *Psalliota campestris* has been the subject of cytological investigations (21, 108, 109) which have demonstrated that in the four spored form each basidiospore is uninucleate at its inception while the two spored form has initially binucleate basidiospores. As would be expected under these circumstances, the two spored form behaves as a balanced heterokaryon similar to *Neurospora tetrasperma*, and is phenotypically homothallic. Colson's (21) account is perhaps the most detailed study of chromosome morphology which is yet available for one of the higher basidiomycetes. Kligman (62, 63) presents a lengthy analysis of nuclear behavior in this species in relation to practical breeding problems. In *Schizophyllum commune*, Ehrlich & McDonough (37) find three haploid chromosomes and report a rather atypical nuclear fusion and reduction. The formation of basidiospores which they find in this organism is quite unlike that reported elsewhere in the basidiomycetes. These unusual details may be due to the difficulty of working with this cartilaginous species, but a reexamination of the material is indicated. Sarazin (107) and Duchaussoy (35) have provided information about the chondriome in the Agaricales, both authors finding a well-developed system of mitochondria. Salmon (105) gives a detailed cytological account of the latex secreting cells of the genera *Lactarius* and *Russula*. Ritchie (99), studying the fixation images produced in the basidia of *Russula emetica* by various acid and basic fixatives, has provided some very useful observations against which other accounts may be measured. He has also noted some curious non-nuclear bodies in the basidia of *Amanita caesaria* (101) which may correspond to the "nebenkern" found earlier by Sass in *Coprinus*. Very little cytological work has been carried out on the resupinate hymenomycetes, although Nobles (82) has briefly reported on the presence of oidia on both primary and secondary mycelia of *Corticium incrustans*, and Skolko (119) has demonstrated that the two spored *Aleurodiscus canadensis* behaves as a balanced heterokaryon. The basidial cytology of a number of polyporaceous species was studied by Bose (9).

Nidulariales.—Brodie (11) has pointed out several interesting aspects of nuclear migration following diploidization in haploid races of the bird's nest fungus *Cyatheus stercoreus*. In most cases diploidization is bilateral, that is, both haploid races of a compatible pair become equally diploidized. However, under some circumstances a unilateral diploidization in which only one of the haploid partners is diploidized may occur. The comparative rates of diploidization of his cultures are most interesting.

In concluding this brief summary of recent contributions to fungal cytology, it may not be amiss to reiterate the urgent need in this field for new methods and approaches, and to point out that, in spite of the incredible number of observations which have been made, we are still pitifully ignorant

of the interrelationship of cellular organelles. The recent upsurge of interest in the genetics and metabolism of microorganisms makes a reexamination of our concepts of fungal cytology imperative.

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ENZYMATIC ADAPTATION IN BACTERIA[1]

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During the past ten years there has been no dearth of general articles on enzymatic adaptation (1, 2, 3, 4), and two critical analyses by Monod (5) and Spiegelman (6) have recently covered much of the material in great detail. A unified treatment demands some consideration of work already reviewed by the two last-mentioned authors; but such data will be dealt with here as summarily as possible, and proportionately more space devoted to the latest developments. Emphasis will be placed on work for which bacteria have provided the experimental material, but references to studies on fungi (particularly yeasts) are unavoidable, since so much of our basic information on the subject has been obtained with these nonbacterial systems.

Enzymatic adaptation may be defined as a change in the enzymatic constitution of an organism that is (*a*) induced by the presence of a specific substance, usually a substrate, in the environment and (*b*) unaccompanied by a change of genotype. Like any short definition of a complex phenomenon, the statement given above is not really adequate to cover all the facts that will be mentioned subsequently, but it serves the essential purpose of segregating the class of enzymatic variations with which we are concerned from the superficially similar class of variations in enzymatic constitution that result from gene mutation followed by selection, and sometimes involve enzymatic adaptation as a secondary event.

So far, enzymatic adaptation has been observed only in bacteria, yeasts, and molds, but the apparent confinement to these microbial groups is merely a reflection of the fact that critical tests have not yet been made to ascertain its occurrence in other living organisms. Such tests would be easy to perform on algae and protozoa, insofar as pure cultures are available. In higher plants and animals, the relative fixity of the internal environment presents an obstacle, but certainly not an insuperable one. The ability of mammals to develop enormously increased tolerances for toxic substances, following chronic exposure, which has been recognized since the classical experiments of Mithradates (7), suggests that a close study of mechanisms of detoxication on the enzymatic level might well reveal the existence of adaptive enzyme formation. Spiegelman (4) has presented arguments in favor of the hypothesis that enzymatic adaptation is a determining factor in ontogeny, but decisive experiments on this point may involve formidable difficulties. An interesting analysis of the possible relationships between the formation of antibodies and the formation of adaptive enzymes has been made recently by Burnet & Fenner (8).

[1] The survey of literature covered by this review was concluded in March, 1951.

Problems of terminology demand some introductory discussion, since the expression "adaptation" is used by biologists with two radically different meanings. Applied to a species, "adaptation" describes modifications of structure or function that fit the species in question for its particular ecological niche (e.g., long necks on giraffes); implicit in this usage is the assumption of genetic change as a mechanism. Applied to an individual organism, "adaptation" describes transient modifications under direct physiological control (e.g., color change in fishes) whose mechanism is clearly nongenetic. This ambiguity has descended into the microbiological realm, where the term "adaptation" has been used by certain workers to describe reversions of biochemical mutants to the wild type, a phenomenon that almost certainly involves back-mutation and selection, as pointed out by Horowitz (9), and thus has nothing to do with "enzymatic adaptation" as defined here. The dangers are obvious, and it can hardly be doubted that the persistent failure of certain authors to differentiate (or sometimes even to grasp the distinction) between enzymatic adaptation *sensu stricto* and mutational changes modifying enzymatic constitution is partly attributable thereto. In order to minimize future confusion, it is proposed that the term "enzymatic adaptation" be applied only to substrate-activated biochemical variations not involving changes of genotype, and that the prefix "enzymatic" be used invariably in such contexts. The abbreviation "EA" is employed in the following pages.

Physiological and Biochemical Aspects of Enzymatic Adaptation

All the known physiological and biochemical facts about EA are compatible with the hypothesis that it represents a special case of protein synthesis: to wit, the synthesis of an enzymatically-active protein by the cell in response to the stimulus provided by the primary substrate. Of course, other interpretations of an observed increase in specific metabolic activity consequent on provision of a substrate are possible: specific changes in permeability, synthesis of required co-factors and the like. These alternative mechanisms have not always been excluded in work on EA, much of which has been conducted by relatively indirect techniques; but there is no positive evidence to support any of them, and an ever-increasing body of evidence, both direct and indirect, to support the hypothesis of a specifically-directed synthesis of enzymatically active protein. In the discussion which follows we will, therefore, make the assumption that synthesis of enzyme protein is the primary event on the biochemical level in EA, using the term "synthesis" in a purely descriptive sense, i.e., without implication concerning the mechanism or the extent of the synthetic reaction concerned.

Methods of measuring enzymatic adaptation.—Since EA involves a specific change in the enzymatic constitution of an organism, it is determined experimentally by measuring changes in the rate of enzymatic activity. Ideally, such measurements should be made under optimal conditions upon solutions of the enzyme concerned, by measuring the disappearance of the

substrate or the formation of the product; but almost none of the work on EA has fulfilled these ideal requirements. Most of the adaptive enzyme systems studied have been intracellular, and since methods for the efficient extraction of enzymes from microbial cells are still very poorly developed, it has usually proved safer in practice to measure the enzymatic activity of intact cells, despite the possible complications arising from the fact that the site of the reaction is thus considerably removed from precise experimental control. Recent experiments by Lederberg (10) indicate that determinations of enzymatic activity upon intact cells may well be subject to major errors. It was found that both the activity and the kinetics of the β-D-galactosidase in *Escherichia coli* were widely different in the intact cell and in a cell extract. After growth on lactose, comparable activities of intact cells and of an extract were 15 and 136 respectively; after growth on maltose, 2.6 and 58. The interpretation of the enormous increases in activity shown by extracts is not yet clear, but the mere demonstration of such increases should serve as a warning against the dangers of placing too much reliance on data obtained exclusively by measurements with intact cells.

An additional complication of work with intact cells possessing full metabolic capacity is the difficulty of measuring directly the rate of an adaptive enzymatic reaction; often an over-all metabolic rate assumed to be governed by the enzyme being adaptively produced is what the investigator actually determines. A few adaptive systems have been studied whose activity can be determined by measuring the rate of formation of the immediate product; examples are the nitratase (11), tetrathionase (12), and formic hydrogenlyase (13) systems in coliform bacteria, which involve respectively the reduction of nitrate to nitrite, the reduction of tetrathionate to thiosulfate, and the splitting of formate to carbon dioxide and hydrogen. When the substrate inducing adaptation is an oxidizable or fermentable compound, the measurements of activity employed have as a rule been highly indirect: in oxidative reactions, over-all rate of oxygen consumption (14) or rate of growth (15); in fermentative reactions, the over-all rate of carbon dioxide production (3) or acid formation (16). Much valuable information has been obtained through experiments involving these highly indirect methods of determining enzymatic activity, and only the general objection to working with intact cells applies, provided that the rate of the adaptive reaction under study is really the factor that limits the over-all rate being measured. It should be kept in mind, however, that this assumption is probably not always true, since at high rates of oxidation the rate of oxygen uptake may well be limited by the hydrogen-transporting capacity of the cell, rather than by the capacity of the adaptive substrate dehydrogenases. A similar consideration applies to the measurement of carbon dioxide production or acid formation in fermentative reactions.

Lastly, it may be noted that work on EA has frequently involved the study of adaptive processes whose biochemical mechanisms were not (and in some cases still are not) known. For example, much of our information

on the general conditions governing EA has been obtained by Spiegelman and his collaborators [summarized in (6)] through the study of the "galactozymase" system in yeasts. Caputto, Leloir *et al.* (17, 18) have recently clarified the biochemistry of galactose fermentation by *Saccharomyces cerevisiae*, which involves the successive intervention of two adaptive enzymes that bring galactose into the glycolytic pathway via galactose-1-phosphate and glucose-1-phosphate. Since the activity of either of these enzymes will limit the rate of galactose fermentation as measured in terms of carbon dioxide evolution, reported data on "galactozymase" reflect net effects on both components of the adaptive system, and not necessarily effects on the enzyme controlling the first step in galactose breakdown.

The role of the inducing substrate.—When unadapted microbial cells are exposed to an adaptively-attacked substrate under conditions otherwise favorable for EA, they manifest (usually after a brief but variable lag) an increased ability to metabolize the substrate. Depending on the particular combination of organism and substrate employed, and also on the prior conditions of cultivation of the cells, the adaptive response may entail either a relative increase in the rate of the pre-existing specific metabolic activity, or an apparent absolute development of specific activity. In the latter event, one cannot assume that unadapted cells were completely devoid of the enzyme system concerned, since the activity may have been too slight for detection by the method of measurement employed. An illustration of this point is provided by recent work on the β-D-galactosidase of *E. coli* (10). The use as substrate of a synthetic galactoside whose aglycone is colored permitted highly sensitive measurements of activity by colorimetry, and revealed the presence in "unadapted" cells of the specific enzyme in amounts that would probably have escaped detection had manometric assay been employed.

If the rate of specific activity during adaptation is plotted as a function of time, the curve obtained usually has a very characteristic sigmoid form. Such curves have been reported for adaptations by many yeasts and bacteria to a wide variety of substrates, and their general interpretation has been much discussed since Spiegelman (3) advanced the hypothesis that the form indicated involvement in the adaptive mechanism of an autocatalytic step. The validity of this hypothesis has been challenged on several grounds. The argument of Monod (5) that an autocatalytic response may be expected, since the adaptive substrate itself serves as the main energy source for the process of adaptation, clearly does not apply to the formic hydrogenlyase system, for which the typical time-activity relationship has been shown, since no energy is obtainable from this enzymatic reaction. However, the argument that a sigmoid curve would be obtained if the time required for adaptation by individual cells in the population varied according to a normal frequency distribution is sound, and almost impossible to test experimentally.

One adaptive process whose kinetics differ radically from the usual

kinetics of EA has been discovered by Pollock (19) in a study of penicillinase formation by *Bacillus cereus*. If unadapted cells are briefly exposed to a very low concentration of penicillin at 0°C., washed, and then placed in phosphate buffer containing glucose, penicillinase is formed at a rate far higher than the basal rate of formation by unexposed cells. Under these exceptional circumstances, there is a lag of approximately an hour, after which enzyme formation proceeds at a strictly constant rate for several hours. When adaptation is conducted in the usual manner in the continuous presence of the substrate, the usual sigmoid adaptive curve results.

The above-mentioned observations on penicillinase production also show that the presence of substrate is a sufficient but not a necessary condition for adaptive enzyme formation, as was first established by Spiegelman & Reiner (20). Brief exposure to a penicillin solution containing 1 unit per ml. gave maximal subsequent penicillinase formation in the formal absence of the substrate, and tracer experiments with radioactive penicillin (21) demonstrated that not more than 5 per cent of the penicillin in solution was fixed by the cells during exposure. Unless one makes the improbable assumption that this minute amount of fixed penicillin is somehow protected from enzymatic destruction within the cell during subsequent adaptation, it follows that a substantial and continuing increase in penicillinase formation must have occurred in the complete absence of the specific substrate. A similar, if quantitatively less dramatic, demonstration of continued adaptive enzyme formation after removal of the substrate was earlier reported by Spiegelman & Reiner (20) for the "galactozymase" system of yeast.

Both these examples of adaptive enzyme formation in the absence of substrate involve a previous exposure of the cells to the substrate in question, even though in Pollock's experiments the exposure was a fleeting one. Wainwright (22) has recently shown in an even more striking manner the nonessentiality of substrate for the formation of an enzyme, nitratase in *E. coli*, which is normally considered adaptive. If *E. coli* is grown in a medium containing a high concentration of amino acids, nitratase activity will attain a high but transient level without any exposure of the cells to nitrate. Under these conditions, maximal nitratase activity per cell is reached at the end of the lag phase, and thereafter falls off rapidly by simple dilution as logarithmic growth proceeds, the total nitratase activity of the whole population remaining almost constant; as a result, cells in the stationary phase (18 hr. old) show very low activity. Systematic experiments with varied sizes of inoculum and concentrations of casein hydrolysate led to the conclusion that the critical factor governing nitratase formation in the absence of substrate is the amino acid concentration per cell: if this is reduced by lowering the concentration of casein hydrolysate or increasing the amount of inoculum, there is a corresponding decline in the peak level of nitratase activity. In the presence of nitrate, variations of inoculum size and concentration of casein hydrolysate affect the maximal titer of enzymatic activity to a far slighter degree, although the nitrogen supply becomes a limiting

factor at low concentrations, as has been observed in many other cases. Thus the effect of nitrate in this system is to maintain nitratase formation under conditions of declining amino acid availability which would cause nitratase formation to cease in the absence of substrate. Nothing could show more clearly the relativity of adaptive phenomena. Not unnaturally, investigators have tended to select for study enzyme systems that show a high dependence on substrate activation, and this selection has in turn tended to obscure the universality of the events involved in EA. Probably all enzyme systems in the cell are adaptive, in the sense that their formation is affected by the presence of substrate; probably no enzyme systems in the cell are strictly adaptive in the sense that substrate is a *sine qua non* for their formation.

Some reference should be made to the influence of substrate concentration on the adaptive process, even though it is almost impossible to interpret the fragmentary available data bearing on this point. Several factors combine to make the evaluation of experiments on the effect of substrate concentration a risky business. In the first place, the mere fact of providing a given substrate concentration in the external environment by no means guarantees that the same concentration will obtain within the cell, and more particularly at the site of enzyme synthesis. Secondly, since the substrate is decomposed by the enzyme system that it is evoking, experiments must be conducted under special conditions if the concentration of the inducing substance is to remain constant in the external environment during the period of induction. The first of these difficulties is inherent in work with intact cells; the second is not, but none of the published data on concentration effects have been obtained under conditions where it was circumvented. For future reference, it may be remarked that a steady concentration of the inducing substance could be maintained either by using a nonmetabolizable substrate analogue (23), or by conducting the experiment in an apparatus similar to that recently described independently by Novick & Szilard (24) and by Monod (24a), which permits the maintenance of a steady state by continuous flow. It must be understood that the data reported below were obtained in experiments where the indicated concentrations are initial concentrations in the environment, which presumably declined at an increasing rate as adaptation proceeded. Monod (25) has reported that the length of the induction period for the growth of unadapted *E. coli* in the presence of several adaptively-attacked sugars is markedly reduced by increasing concentration up to a value of 1×10^{-3} *M*, although saturation of the enzyme systems concerned is complete at concentrations of the order of 2×10^{-5} *M*. Data on the nitratase system of *E. coli* (22) obtained by measuring rates after exposure to the substrate for 1 hr., show a rapid increase in activity with an increase in concentration up to 5×10^{-4} *M*; further increases in substrate concentration gave constant activities. The substrate affinity of this enzyme is not known. The penicillinase system in *B. cereus* appears to be activated by strikingly low substrate concentrations; measurements of

rates after 2.5 hr. exposure to the substrate showed a detectable effect at 8×10^{-9} *M*, and full activation at 2×10^{-7} *M* (19). Again, the substrate affinity of the enzyme is unknown.

In the past, the assumption has often been made that induction by a single primary substrate results in the formation of a single enzyme. This is probably true for a few adaptive systems, involving substrates whose entire metabolism requires only a single step-reaction, or ones that can be brought through a single adaptive step into metabolic pathways controlled by the pre-existing enzymes of the cell, but in most instances of this sort the simplicity of the enzymatic step is inferred rather than experimentally verified. The adaptive response is often a more complex one, since for many adaptively-attacked substrates two or more successive step-reactions, each under adaptive control, are required in order to bring the primary substrate into the central metabolic machinery of the cell. A simple example already mentioned is the adaptation of yeasts to ferment galactose, which requires the formation of galactokinase, catalyzing the reaction: galactose + ATP →galactose-1-phosphate+ADP; followed by the formation of galactowaldenase, catalyzing the reaction: galactose-1-phosphate→glucose-1-phosphate. The latter compound is a member of the main glycolytic pathway. Since the substrate for galactowaldenase is the product of the primary phosphorylation of galactose, both enzymes will be formed when the cell is exposed to galactose. Numerous other, more complex, examples of the same phenomenon are known, and the technique of simultaneous adaptation for the biochemical analysis of adaptively-controlled metabolic pathways (14) is based upon it. The biochemical aspects of simultaneous adaptation will be discussed in a later section; the point to be stressed here is that in such cases one deals with an adaptive situation that is far from simple, since the external provision of a single compound has the effect of exposing the enzyme-forming machinery of the cell to a multiple stimulus (primary substrate plus adaptively-attacked intermediates) whose magnitude may not even be realized. Insofar as studies on kinetics and on extrinsic factors governing adaptation are concerned, such systems clearly do not provide ideal material, and this implies the desirability, before undertaking such studies, of understanding in biochemical terms the nature and extent of the adaptive reaction to be investigated.

Simultaneous adaptations also have considerable bearing on the question of the specificity of the adaptive response. Before assuming that the synthesis of an enzyme has been evoked by a compound other than the true substrate, one must exclude the possibility that the true substrate has been produced as an intermediary metabolite from the apparently nonspecific inducing compound. An experiment reported by Mirick (26) illustrates the point. In a detailed investigation of the conditions governing formation of the enzyme systems responsible for the oxidation of aminobenzoic acids by an unidentified soil bacterium, he observed one marked exception to the generally very great specificity of substrate activation: the adaptive system

responsible for the oxidation of anthranilic acid could be fully activated by exposure to tryptophan. This might still stand as an example of nonspecific activation had it not been shown subsequently by Suda *et al.* (27) that anthranilic acid is an intermediate in the oxidation of tryptophan by certain bacteria.

Many of the other reported nonspecific activations involve formation of an enzyme in response to its reaction product. Some examples are: lactase produced in response to galactose (28); the specific hydrolase for type III pneumococcal polysaccharide in response to aldobionic acid (29); benzaldehyde dehydrogenase in response to benzoic acid (14); and succinic dehydrogenase in response to fumaric acid (30). In studies on penicillinase formation (19), Pollock found that apparent activation by the reaction product, penicilloic acid, was caused by minute contamination with penicillin, and he has expressed scepticism about other claims for nonspecific activations of this class. However, they have been observed with so many systems that contamination of the chemicals employed is not likely to be the universal explanation. Insofar as the enzymatic reactions concerned are readily reversible, activation by the product can be considered simply as an extension of the phenomenon of simultaneous adaptation (31).

Other classes of nonspecific activations are extremely rare, as a perusal of the data collected by Monod (5) will show. A very interesting example has been discovered recently by Spiegelman *et al.* (23): the formation of "maltozymase" by yeast in response to α-methylglucoside, an unmetabolizable substrate analogue possessing in common with maltose the α-glucosidic linkage. A systematic search may well reveal other similar cases.

There have been several reports showing that adaptive enzymes can be formed in response to the presence of the substrate even under conditions that preclude exercise of their enzymatic function. An elegant demonstration of this was made by Bellamy & Gunsalus (32) as a by-product of studies on the conditions for the production by *Streptococcus fecalis* of tyrosine decarboxylase. This enzyme requires pyridoxal phosphate as a coenzyme. *S. fecalis* cannot synthesize pyridoxine, but can nevertheless be grown in its absence, provided that the medium contains an excess of alanine. The cells from such a culture contain an abundance of the apoenzyme of tyrosine decarboxylase, which is completely nonfunctional as a consequence of the absence of pyridoxal phosphate from the cells. The presence of the apoenzyme is readily demonstrable in cells or extracts therefrom when the coenzyme is added. Spiegelman *et al.* (33) have demonstrated the adaptive formation of "maltozymase" in yeast under conditions precluding enzymatic function; this was achieved by exposing the cells to maltose at a pH of 8.5.

Extrinsic physiological factors affecting EA.—A variety of extrinsic factors greatly affect the rate and extent of EA. They include nitrogen supply, energy supply, pH, and temperature.

Many microorganisms cannot adapt at all unless a utilizable external nitrogen supply is provided, although there is no way of predicting a priori

whether a nitrogen supply will be essential for any given system. Among bacteria, for example, it has been a common experience that most adaptations by *E. coli* will not proceed in the absence of an external nitrogen source; however, Monod (34) has recently reported that a strain employed by him will adapt to a small extent without one. *Pseudomonas fluorescens* can readily adapt to a wide variety of substrates without an exogenous nitrogen source (14). Presumably the variability in this respect is attributable to differences in the amount or mobilizability of the endogenous nitrogen supplies. Even when an external nitrogen supply is dispensable, however, adaptation may be more rapid and proceed to higher levels if a readily utilizable nitrogen source is provided. Furthermore, in yeasts it has been shown that competitive effects between enzyme systems during adaptation are greatly reduced by the provision of an external nitrogen source (35).

The energy required for the initiation of adaptation can in most cases be supplied by oxidation or fermentation of the endogenous reserves, but it has been clearly shown that provision of a readily oxidizable or fermentable substrate will greatly accelerate the adaptive process under conditions where the endogenous reserves are either limiting in amount, or unutilizable (36).

The influence of temperature upon adaptation has been examined with care only for the tetrathionase and nitratase systems in coliform bacteria (11, 37, 37a). For the tetrathionase system the rate of adaptation was maximal at 35 to 37°C., which is roughly the optimal temperature for growth. Adaptation was completely inhibited at 41°C., although the reaction rate of tetrathionase was considerably higher at this temperature than at 37°C. Formation of nitratase was much less affected by elevated temperatures.

The effect of the pH of the environment during growth on adaptive enzyme formation by *E. coli* has been studied in great detail (38, 39); this work was extensively summarized in a review by Gale (2). The most striking effect of variations in external pH was manifested in the synthesis of the amino acid decarboxylases and deaminases. In general, amino acid deaminases were formed in large amounts under alkaline conditions, and in negligible amounts under acid conditions, while formation of amino acid decarboxylases followed a reverse pattern. This response to variation of external pH would have the effect of shifting the external pH towards neutrality, by favoring decarboxylation of the amino acids in the environment under acid conditions, and deamination under alkaline conditions; it was interpreted on teleological grounds by Gale & Epps, " . . . a change in external pH is followed by an alteration in the enzymic constitution of the cells such that an attempt is made to counter the external change . . . " (39). A more scientific explanation, also advanced by them, is that in the case of these amphoteric substrates the substituent removed by enzyme action must be in undissociated form in order to evoke enzyme formation. Under alkaline conditions, the amino group is undissociated (formation of deaminases); under acid conditions the carboxyl group is undissociated (formation of decarboxylases).

Selective inhibition of EA.—EA can be completely prevented by a variety of physical, chemical, and biological treatments that have little or no effect on the pre-established catabolic activities of the cell. Maintenance at a supra-optimal temperature as a means of inhibiting EA (37a) has already been mentioned in the preceding section. At least with the coliform bacteria used to study temperature effects, a temperature sufficiently high to abolish the adaptive process causes no impairment of viability; in this respect, temperature inhibition is probably unique, although careful studies of viability following treatment with other selective inhibitors have not been reported. Another physical agent remarkably effective as a selective inhibitor of EA is ultraviolet irradiation; the inhibition is photoreactivable (40, 41). A variety of chemicals at appropriate concentrations can also be employed: sodium azide (42, 43), sodium arsenate (44), 2,4-dinitrophenol (45), methylene blue (46), 8-hydroxyquinoline (47), streptomycin (48), and aureomycin (49). In *E. coli* (and probably in other bacteria as well, though this has not been tested) virus infection blocks EA (50).

In view of their diversity, it is likely that these inhibitors exert their effect on the adaptive process at a number of different points, and a careful study of the respective modes of action might throw considerable light on the inner mechanism of EA. Among the chemical inhibitors, azide, 2,4-dinitrophenol, and methylene blue are known to be inhibitors of assimilation at the concentrations effective in blocking adaptation.

Ultraviolet irradiation has no effect on oxidative assimilation in doses that prevent adaptation (41). Its mode of action may, therefore, be a different one from that of the chemical inhibitors discussed above. The action spectrum for inhibition of adaptation has recently been determined (51), and follows very closely the absorption spectrum of nucleic acid. This observation suggests that the nucleic acids of the cell may play a direct part in the adaptive process, and perhaps lends support to the claim, not yet substantiated by detailed data, that extracts containing ribonucleic acid stimulate EA in yeast (52).

Direct evidence for the synthesis of enzymes during enzymatic adaptation.—While many of the observations on factors affecting the adaptive process are consonant with the hypothesis that the primary event is the synthesis of enzymatically-specific protein, they are not an adequate substitute for the direct proof of this hypothesis. Fortunately, such proof is now available for a number of adaptive systems. It was first obtained by Doudoroff *et al.* (53), who showed that the enzyme sucrose phosphorylase could be obtained from preparations of *Pseudomonas saccharophila* grown on sucrose, but not on other carbohydrates. Some years later, Spiegelman *et al.* (54) proved the apoenzymatic nature of adaptation by yeast to ferment galactose. More recently, direct proofs for the synthesis of enzymatically active protein during EA have been obtained for a variety of adaptive systems in bacteria: amylomaltase in *E. coli* (55, 56), hexokinase and sucrose phosphorylase in *Pseudomonas putrefaciens* (57, 58), enzymes oxidizing many aromatic sub-

strates in *Pseudomonas fluorescens* (31, 59, 60). In no case where a search for such proof has been conducted adequately has a failure to obtain it been reported.

Competitive effects in adaptation.—The phenomenon of EA shows that the microbial cell does not at any given time fully express its potential enzyme-forming capacities, and that a certain selectivity, over and above that conferred by the genetic endowment, enters into the process of enzyme formation. Since the expression of a latent enzymatic capacity can be evoked by external provision of the specific substrate, it might be anticipated that simultaneous exposure to several different substrates would bring into play competitive interactions, the severity of which would be determined by the enzyme-forming capacity of the cell and by the quantity of raw materials available. We owe to Monod (5, 15, 61, 62, 63) the first precise demonstration and most exhaustive analysis of such competitive effects. During the course of his studies on the nature of bacterial growth, Monod observed that bacterial cultures (*E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*) in media that contained limiting amounts of two carbohydrates sometimes exhibited two cycles of growth, separated from one another by a lag phase (the phenomenon of "diauxie"). A closer analysis revealed that each cycle involved the exclusive utilization of one member of the pair of carbohydrates, and that the intervening lag represented the time required by the cells for adaptation to the previously unutilized member of the pair. Systematic tests showed further that for any given bacterial strain the utilizable carbohydrates could be classified into two groups, "A" and "B"; any pair selected from "A" gave rise to a normal one-step growth curve, whereas any pair consisting of an "A" and a "B" sugar produced diauxie, utilization of the "B" sugar being prevented until the supply of the "A" sugar is exhausted. Even if the inoculum consists of cells grown previously in the exclusive presence of the "B" sugar, growth in an A+B mixture follows the usual diauxic course. The diauxic phenomenon shows in the clearest and most absolute manner an extreme example of competitive enzyme formation, activated by the external provision of two utilizable substrates and occurring despite the presence of an ample nitrogen supply.

Similar phenomena were observed independently by Spiegelman & Dunn (35) in yeast. When galactose and maltose are added simultaneously to a resting suspension of yeast devoid of an external nitrogen supply, adaptation to both compounds takes place at first, but as time proceeds "maltozymase" activity passes through a peak and then declines to a very low value, while "galactozymase" activity increases in the normal manner, finally reaching a value identical with that achieved in the presence of galactose alone. In the presence of an external nitrogen supply, the suppression of "maltozymase" activity, although still measurable when compared to a control with maltose alone, is much less severe. It was also found that in the absence of an external nitrogen supply, adaptation to galactose can result in a slight depression of "glucozymase" activity, despite the fact that glucose fermenta-

tion is not in the usual sense "adaptive," the cells possessing this capacity to a high degree irrespective of the conditions of cultivation. This experiment is of particular value in demonstrating that no microbial enzyme system can be considered wholly immune to the competitive interplay that is shown with particular sharpness by the more strictly adaptive systems.

Considered purely on the enzymatic level, competitive effects can be interpreted in two ways. In certain cases there may be an actual interconversion of enzymes, probably through inactive intermediates. Such interconversions might be expected to manifest themselves under conditions of acute competition, and it is to be noted that the data cited above on competitions in the absence of an external nitrogen source are strongly suggestive of the existence of interconversions. The other possibility is the suppression of enzyme formation, which would lead in a resting population to the maintenance of a fixed level of activity of the weaker system, but in a growing population would soon reduce the level of activity per cell to a negligible value by simple dilution. Simple suppression, rather than interconversion, might well occur when the nitrogen supply is not a limiting factor, and is certainly not excluded as a possible mechanism in the experiments of Monod.

Deadaptation.—After what has already been said, the subject of deadaptation can be dismissed very briefly. The term refers to the loss of specific activity by an adapted population following removal of the activating substrate. The most detailed analysis of the phenomenon has been made by Spiegelman *et al.* (20, 52) working with yeasts, and their data—obtained for the most part with resting cells—suggest that deadaptation occurs by active conversion of the adaptive system, presumably to other enzymes. Wainwright & Pollock (47), on the other hand, have found that with growing cultures of *E. coli*, deadaptation of the nitratase system takes place by simple dilution. There is not necessarily any contradiction between these two sets of findings; as stated above, both interconversion and suppression may operate to reduce the level of specific enzymatic activity, and their relative importance in any given case may well be determined by the experimental conditions.

Genetic Aspects of *Enzymatic Adaptation*

In the introductory definition of EA, it was stated that the phenomenon takes place without a change of genotype. It is thus necessary to distinguish on the biological level between EAs and other observed biochemical acquisitions of function that are primarily caused by mutational change.

The experimental distinction between enzymatic adaptation and mutation.—Since EA occurs against the background of a constant genotype, the overwhelming majority of cells in a microbial clone can respond to the specific stimulus provided by the inducing substrate. A spontaneous mutation that confers the ability to metabolize a substrate will, on the other hand, exist in a very small minority of the cells in a clone, unless that clone has been allowed to grow under conditions strongly selective for the mutant (e.g., in

the presence of the specific substrate). Consequently, the experimental distinction between EA and gene mutation as primary causes of observed acquisitions of function can be made by determining whether, in a microbial population grown in the absence of the substrate concerned, a vast majority of the cells are potentially capable of metabolizing the substrate, or only a very small minority. Provided that EA takes place in the absence of a nitrogen source, the test can be made by exposing a resting population to the substrate and looking for the gross development of specific metabolic activity. If EA can occur only under conditions that permit growth, the problem of selection has to be taken into account. A mere doubling of the population, if entirely the result of mutant growth, would be ample to cause the gross appearance of specific metabolic activity. Here, however, a temporal factor enters. Let us consider a population of 10^8 cells, of which 10^3 are mutants capable of selective growth in the presence of the substrate. If we set 1 per cent of the total population as a safe level below which biochemical activity of the mutant would be imperceptible, approximately ten generations of mutant growth would be required to reach this level; even with a very short generation time (20 min.), over 3 hr. would elapse before the danger point was approached. Consequently, even if growth is possible under the conditions of the test, the attainment of a high level of activity within a few hours is *prima facie* evidence for EA. When a longer lag occurs before specific biochemical activity is evidenced, the presumption of a primary mutational change followed by selection must be entertained. In such circumstances, the decisive test to distinguish between EA and mutation will be the fluctuation test of Luria & Delbrück (64).

Such lengthy discussion of this very simple methodological point might appear unnecessary, were it not that confusions of mutational and adaptational phenomena are still frequent. For example, Jackson & Hinshelwood (65) have recently published experiments which they interpret as showing that certain well-known mutational acquisitions of function in bacteria, including the acquisition of the ability to ferment lactose by a mutabile strain of *E. coli*, are "adaptive." After dismissing the fluctuation test as invalid with the allegation (not supported by experimental evidence) that the lag periods in a set of identical cultures grown under near-optimal conditions will vary widely, they proceeded with an experimental analysis by the comparison of plate and dilution counts in differential media. This method was used by Lewis (66), many years ago, to demonstrate the infrequency of lactose positive cells in clones of *E. coli mutabile*. His data (which were not critically discussed by Jackson & Hinshelwood) showed counts on nutrient agar or mineral glucose agar approximately 50,000 times those on mineral lactose agar. The success of the method depends, of course, on using a rigidly selective medium to demonstrate mutants; if appreciable parental growth can occur at the expense of impurities, mutations in the test plate become possible. Jackson & Hinshelwood gave no data to show that this vital point had been adequately controlled by them; consequently the fact that they

observed counts after 62 hr. on lactose agar of the same order as those after 14 hr. on glutamate agar does not permit the conclusion that every cell in the inoculum was potentially capable of utilizing lactose. The remainder of their experiments are open to similar criticisms.

The influence of genotype on enzymatic adaptation.—The fact that EA occurs without a change of genotype does not imply, of course, that the adaptive process lacks a genetic component. To the contrary, all available evidence indicates that the potential capacity of an organism to produce an adaptive enzyme is subject to strict genetic control. However, phenotypic expression is achieved only when the relevant substrate is present in the environment.

The influence of genotype on adaptive enzyme formation has been studied most extensively in yeasts, where the existence of sexuality, coupled with the convenient segregation of the products of meiosis in the ascospores, makes mendelian analysis relatively easy. Winge and his collaborators (67, 68, 69) and the Lindegrens [summarized in (70)] have demonstrated that the capacity to ferment adaptively a number of carbohydrates is gene-controlled. For some sugars, only a single gene is apparently involved; for others, any one of several nonallelic genes may confer fermentative ability. In hybrids resulting from crosses between fermenters and nonfermenters, fermentative ability is always dominant. Genetic analyses on the segregants from hybrids have not infrequently revealed unexpected ratios, the interpretation of which has led to much discussion. Lindegren has advanced a series of highly novel hypotheses, while Winge has attempted to explain such findings in classical genetic terms. The recent report of Roman, Hawthorne & Douglas (71), who found that the four ascospores in an aberrant ascus were diploid, suggests that many of the unusual ratios observed in the past may have resulted from polyploidy, and provides strong support for the position adopted by Winge.

Until recently, the evidence for genetic control of adaptive enzyme formation by bacteria was less conclusive, owing to the presumed absence of sexuality in these organisms. In several instances it has been shown that bacterial mutations involving the acquisition of a new biochemical property reflect the acquisition of the capacity to produce a specific adaptive enzyme: the most convincing demonstrations of this have been made by Monod & Audureau (72) for the mutabile mutation of *E. coli*, and by Klein & Doudoroff (58) for the mutation of *P. putrefaciens* to utilize glucose. The discovery of genetic recombination in *E. coli* by Lederberg (73) made it possible to study directly the genetic control of adaptive enzyme formation with at least one strain of this bacterial species. Lederberg's analyses of the recombination of many biochemical characters, including the characters of lactose and maltose fermentation, which are both adaptive, indicate the existence in *E. coli* of a single linkage group, with the genes arranged in linear fashion. Normally *E. coli* is haploid in the vegetative state, but Lederberg has described (74) an unstable diploid heterozygote. Among the characters for

which this hybrid is heterozygotic is lactose fermentation, and both lactose positive and lactose negative haploid segregants are formed from it. As in the case of yeasts, the adaptive capacity to ferment a carbohydrate is dominant.

While it is well established that specific genes are required for the initiation of adaptive enzyme formation, this does not necessarily prove that the nuclear genes are also required for the maintenance of enzyme production, once it is induced by the presence of the specific substrate. Some years ago, this question was examined by Spiegelman, Lindegren & Lindegren (75), who studied the influence of the specific substrate on the segregation of the character for melibiose fermentation in a heterozygotic yeast. Their data appeared to show that the normal 2:2 segregation ratio of fermenters to nonfermenters could be altered to a 4:0 ratio by the presence of melibiose, suggesting that the nuclear gene is unessential for the continued production of the enzyme. Unfortunately, subsequent attempts to confirm this very interesting observation have been unsuccessful (70). More recently, Monod has conducted analogous experiments with Lederberg's strain of *E. coli*, using lactose as the test compound (34). His data show that the presence of lactose has no effect on recombination ratios. Thus, the somewhat scanty evidence available at the present time suggests that the genetic component is essential both for initiation and for maintenance of adaptive enzyme formation.

The phenomenon of "long term adaptation."—The expression "long-term adaptation" was introduced by Winge & Roberts (68) to describe a peculiar response to substrate shown by *Saccharomyces chevalieri*. Normally, a yeast possessing the genetic endowment for the fermentation of a given sugar will produce carbon dioxide vigorously within 24 hr. after inoculation into a medium containing the sugar concerned (the usual "short-term adaptation"). When *S. chevalieri* is inoculated into galactose media, carbon dioxide production is imperceptible for the first day or so, and only becomes vigorous after five or six days; once galactose-fermenting ability has been developed, it can be maintained at a high level by continued transfer of the adapted strain through galactose media, but is usually lost rapidly after transfer to a medium devoid of this sugar. By making crosses to *S. cerevisiae*, which ferments galactose in the usual manner, Winge & Roberts showed that the slow galactose character of *S. chevalieri* behaved as if controlled by a single recessive gene (g_s), allelomorphic with the dominant gene for rapid fermentation (G) present in *S. cerevisiae*. They concluded that slow galactose fermentation is caused by a low rate of enzyme synthesis in response to the presence of the substrate. Their experiments were not conducted in such a manner as to exclude rigorously the possibility of mutation and selection, although as they later pointed out (69), an explanation in these terms would involve some highly improbable assumptions.

A close analysis of the phenomenon has recently been made by Spiegelman *et al.* (76). From their findings, it appears that "long-term adaptation"

is indeed an adaptational event, but of an entirely new and unexpected kind. When a slow-fermenting stock is plated on an indicator medium containing galactose, two kinds of colonies develop: a few large colonies, consisting of cells capable of immediate rapid fermentation of galactose ("positives"), interspersed with many small colonies not possessing this capacity ("negatives"). When plated on fresh indicator media, however, the negatives can be shown to contain a high proportion of potential positive cells (10 to 60 per cent). Further experiments showed that negative cells have an inhibitory effect on positives, which explains why a high proportion of potential positives in a negative colony does not cause the colony to have the positive appearance. Since the proportion of positives to negatives in slow-fermenting stocks is of the order of 1:1000, the long delay in the onset of fermentation in liquid galactose media must also be caused by an initial inhibition of the positives by negatives. At first sight, these findings seem to imply that the basis of "long-term adaptation" is the selection of positive mutants, complicated by inhibitory effects. However, the additional data obtained by Spiegelman *et al.* cannot be interpreted on such an hypothesis. Firstly, a series of fluctuation tests gave observed variances far below those to be expected if mutation and selection were operative, which suggests that the substrate galactose can induce positiveness in a small and uniform proportion of the cells. Secondly, positive populations grown in the absence of galactose were found to undergo mass reversion to the negative phenotype. The manner of occurrence of this reversion is extremely constant, provided that recently-developed positive clones are studied. After inoculation into a galactose-free medium, the positives grow at a steady rate for five generations; at this point, their growth rate falls to zero, and at the same time large numbers of negative cells appear, which grow therafter at the rate previously characteristic of the positives. This burst of negatives always occurs between the fifth and sixth generation; calculations show that it cannot be explained in terms of the known growth rate of negatives, but is entirely consonant with the assumption that the positive cells at this time start to produce negative buds. Clearly, much more work will have to be done before a final interpretation is possible, but the conclusion of the authors that a transmissible cytoplasmic factor determines phenotypic positiveness appears to be the simplest explanation for the observed facts.

The Use of *Enzymatic Adaptation* as a Tool in Biochemical Research

It has already been mentioned that an organism may respond to the provision of a single, adaptively-attacked substrate by producing a sequence of enzymes, acting on the primary substrate and on the adaptively-attacked intermediates produced therefrom. The phenomenon can be used as the basis for a refined type of kinetic analysis to determine the nature of the intermediates that lie on an adaptively-controlled metabolic pathway, a discovery made independently by several workers (14, 27, 30). This kind of analysis

has been termed "simultaneous adaptation" (14) and "successive adaptation" (27). The principles of the method can be illustrated by considering a hypothetical metabolic pathway, each step of which is catalyzed by a specific adaptive enzyme:

$$\underset{}{A} \xrightarrow[E_A]{} \underset{}{B} \xrightarrow[E_B]{} \underset{}{C} \xrightarrow[E_C]{} \underset{}{D} \xrightarrow[E_D]{} \underset{}{E} \xrightarrow[E_E]{} \text{etc.}$$

Cells potentially capable of carrying out these reactions will be devoid (for all practical purposes) of the relevant enzymes if they have not been exposed by the conditions of growth to compounds A, B, etc. When placed in contact with A, such cells respond by producing E_A, catalyzing the step-reaction A→B. The formation of B will in turn provide the necessary activation for the formation of E_B, and so on. Thus, cells fully adapted to dissimilate A will also be full adapted to dissimilate B, C, D, etc. If one adapts the cells to any intermediate in the chain (say, C), they will then also be adapted to the later intermediates D, E, etc., but not necessarily to the earlier ones; although it not infrequently happens that adaptation to an intermediate C will cause back-adaptation to the immediately preceding member of the chain, B (14, 30, 77), presumably by virtue of the fact that the reaction is readily reversible (31). Consider, however, the case of a compound X, which may appear on chemical grounds to be a possible intermediate in the dissimilation of A, and which is likewise potentially attackable by a specific adaptive enzyme E_X. In view of the known high specificity of the adaptive response, it is extremely improbable that cells specifically adapted by exposure to A will also be adapted to X, if X is not a member of the reaction chain. Thus, by adapting an organism to a given primary substrate, and then analyzing its adaptive patterns with respect to postulated intermediates, evidence can be obtained as to which of these postulated intermediates are actually operative. It is, of course, also necessary to test the behavior of "unadapted" cells grown in the absence of all the compounds tested, in order to make certain that the relevant enzymes are not always present in the cells. The technique can be applied even in cases where adaptive control is only partial, provided that exposure to A markedly increases enzymatic activity over the basal level in "unadapted" cells (78).

The validity of the biochemical inferences drawn from such experiments rests on two assumptions, which may not always be correct: rigid specificity of adaptive response, and free permeability of the cell to all compounds tested. If the first of these assumptions is incorrect, false positive conclusions are possible: if the second, false negative ones. The fact that enzyme formation can be evoked by unmetabolizable substrate analogues (23) shows that at least under these exceptional circumstances the first assumption is untrue. Furthermore, there is one report of the activation of the first few steps in a reaction sequence by a metabolically unrelated compound (14); in this particular case, the lack of adaptation to later steps in the sequence prevented a false interpretation. It has also been observed (31) that activation of

the enzymes operative in one reaction chain may greatly shorten the time required for adaptation to a metabolite lying on a related pathway, but here, too, the existence of a lag, even though abbreviated, made correct conclusions possible. The assumption of free permeability is probable a more serious cause of error (79), but all metabolic work conducted upon intact cells involves this particular difficulty, and in practice the misinterpretations that stem from it are usually perceived only as a result of subsequent experiments with cell-free systems.

One alleged proof of the invalidity of the general method, offered by Campbell *et al.* (80), is based on faulty logic. These authors observed that

TABLE I

BIOCHEMICAL INVESTIGATIONS CONDUCTED IN WHOLE OR IN PART BY THE ANALYSIS OF ADAPTIVE PATTERNS

Organisms	Metabolic process studied	Reference
Azotobacter agilis	Oxidation of acetate and Krebs cycle compounds	(30)
Escherichia coli	Oxidation of acetate and Krebs cycle compounds	(78)
Escherichia coli	Oxidation of uronic acids and pentoses	(84)
Achromobacter sp.	Oxidation of phenol and Krebs cycle compounds	(83)
Pseudomonas fluorescens	Oxidation of aromatic compounds	(14, 85, 86)
Pseudomonas spp.	Oxidation of tryptophan	(27, 77, 87, 88)
Pseudomonas sp.	Oxidation of tyrosine	(89)
Rhodopseudomonas gelatinosa	Oxidation (photosynthetic) of isopropanol	(90)
Clostridium acidi-urici	Fermentation of purines	(91)
Azotobacter vinelandii	Pathway for fixation of molecular nitrogen	(92)

Pseudomonas aeruginosa adapted to oxidize glucose is not adapted to oxidize either 2-ketogluconate or acetate; yet, under certain circumstances, both acids can be detected in the medium during growth on glucose. The fact of accumulation was assumed to provide incontrovertible evidence that both compounds lay on the direct pathway of glucose oxidation, a conclusion that is superficially tempting, but not necessarily sound. It is entirely possible that neither acid as such is an intermediary metabolite, but that the true intermediates are labile derivatives (e.g., phosphorylated compounds), which can in part be converted to the free acids and excreted into the medium. All recent work on acetate metabolism (81) is in accord with this alternative interpretation, and shows that free acetate lies entirely outside the main pathway of terminal oxidation; the studies of McNair Scott & Cohen (82) suggest a parallel situation with respect to 2-ketogluconate.

Actually, the work of Campbell *et al.* can be interpreted as a strong argument against the direct participation of acetate and 2-ketogluconate in the pathway for oxidation of glucose by fluorescent pseudomonads. A second, purely theoretical objection to the method offered by these authors is based on a misunderstanding of the kinetics of the steady state.

Lack of space precludes detailed discussion of the biochemical investigations conducted, in whole or in part, by the analysis of adaptive patterns. The relevant information is summarized in Table I.

EA has a second biochemical application, so far not much developed; namely, as an analytical tool. Cohen & Raff (93) have recently worked out in detail the use of specifically-adapted strains of *E. coli* for the individual analysis of gluconic acid, D-arabinose, and D-ribose. Specific bioassays could be made by determining growth at the expense of these compounds, or by manometric determination of the acid formed as a result of their fermentation. The manometric assay permitted quantitative estimations on amounts of 200 μg. Analogous manometric assays with specifically adapted cells of *P. fluorescens* have been used in our laboratory to determine small amounts of mandelic and benzoic acid. Such methods, while tedious and not highly accurate, could undoubtedly have many applications in the study of problems for which no suitable chemical methods of analysis are available. They represent a logical extension of the principle of microbial degradative bioassays first developed for the analysis of sugars by Kluyver some 30 years ago (94). In the long run, however, it is likely that they will be replaced by the more refined method of using cell-free enzyme preparations, which would eliminate many of the variables inherent in assays with living cells. The manipulation of adaptive patterns prior to the extraction of specific enzymes will be an important factor in developing such enzymatic assays.

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ADAPTATION IN FUNGI[1,2]

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INTRODUCTION

Interest in the problem of adaptation in fungi has been centered on directed changes in pathogenicity, acquired ability to utilize organic compounds, and increased tolerance to toxic materials. The common denominator of all these processes is the acquisition of a new function by continued exposure to a specific factor introduced into the environment.

In addition to its intrinsic scientific value, adaptation has considerable practical importance. There are many reports that the virulence of fungi may be profoundly changed by passage through living host-plants. By this method, relatively weak races of parasitic fungi supposedly could adapt themselves to previously resistant or immune hosts and become destructive pathogens; even saprophytic fungi could gradually be educated to become active parasites if given the proper stimulus. Obviously, if fungi could be educated readily to attack resistant varieties of crop plants, breeding for disease-resistance would be futile.

The industrial mycologist who searches for cheap energy sources in fermentations and vitamin or antibiotic productions frequently has found adaptation to a particular substrate economical in time and labor. Screening procedures for fungicides, based on rates of growth in the presence of the toxicant, are subject to serious error if adaptation is ignored.

It is recognized that the observed behavior may be attributed either to the selection of variants from mixed cultural populations or to a temporary or reversible change not involving alteration in the gene complement. Despite the fundamental difference in the nature of these two processes, few investigators distinguish between them, perhaps in part because of experimental difficulties. Since selection is a distinct concept already established, with genetical connotations, in other biological sciences it is logical to apply the term adaptation only to temporary changes not involving alteration in gene complement. Such a procedure has the advantage of indicating both the phenomenon and the mechanism responsible for it, and will be used in this review in order to avoid ambiguity.

Restriction of the term adaptation to reversible changes imposes ad-

[1] The survey of literature pertaining to this review was completed in December, 1950.

[2] Paper No. 746, Misc. Journal Series Article, Minnesota Agricultural Experiment Station.

ditional experimental burdens in order to justify its use in any given instance. Genetic analysis of multicellular fungus colonies poses problems not encountered in bacterial population studies, problems which are augmented if the fungus is dicaryotic or multinucleate. Genetic variation in asexually propagated cultures is, then, a consequence of nuclear dissociations or reassociations (heterocaryosis) in addition to classical chromosomal aberrations and point mutations.

PARASITIC ADAPTATION

Changes in Virulence on Culture

Certain pathogens may have their metabolism altered materially when grown on artificial nutrients. Rathbun-Gravatt (75) and White & McIntyre (102) found that pathogenicity was influenced by the nutrient media on which the parasite grew. Age of culture and temperature at which inoculum was grown also are factors that may influence the initial parasitic ability of a pathogen. Perhaps the organisms lose certain enzymes or growth-promoting substances which they cannot synthesize in an artificial substrate and this may account for the apparent loss of virulence.

Phenotypic characters acquired while on a nutrient medium can frequently be changed by passage back through the host. Dastur (27) restored the spore-producing power of a variant from *Glomerella* sp. by doing so. Coons & Larmer (25) found that *Cercospora beticola* Sacc. tended to revert to the original type after passage through the host, whereas Caldis & Coons (16) could not restore the fruiting capacity of a variant line from *Cladosporium fulvum* Cke. by passage through tomato. Christensen (18), Keitt & Langford (53), and Ullstrup (96) found that cultures of several pathogenic fungi were not altered by passage through the host plant.

La Rue (54), Christensen & Davies (21, 22) and others attributed the loss in virulence of pathogenic fungi in culture to mutation; the mutant frequently was able to thrive better in culture and thus replaced the parental line. Although most mutants observed in culture were less virulent than their parent, some were decidedly more virulent. Hiroe (46) found the same in *Ophiobolus miyabeanus* Ito & Kuri. and Das Gupta (26) obtained similar results with *Cytosporina lubidunda* Sacc. Brown (12) and Mitter (63) reported that variants of *Fusarium* sp. derived from a single spore may actually transcend the species limits. If new virulent races arise in culture, it seems reasonable to assume they also arise in living hosts.

Burkholder (14) found that the virulence of *Fusarium martii phaseoli* Burk. had been greatly reduced during six years on artificial media. The original virulence of the pathogen was restored after three successive passages through the host. Bonar (6) proved that a newly isolated line of *Brachysporium trifolii* Kauf. was much more pathogenic than one that had been in culture continuously for more than two years.

Barring mutation, there is much evidence that fungi do not always lose

their virulence even when grown on artificial media for long duration or have their phenotype changes as a result of passage through the host. For instance, Keitt & Langford (53) passed three haploid lines of *Venturia inequalis* (Cke.) Aderh. through the leaves of the McIntosh apples four successive times and obtained no apparent changes in virulence. The senior author (20) has obtained similar results with certain monosporous lines of *Fusarium lini* Boll., *Gibberella zeae* Schwe., *Collecotrichum linicola* Peth., and Laf., and *Helminthosporium sativum* P. K. and B. Likewise, Bailey (2) passed *Cladosporium fulvum* Cooke through incompatible tomato tissue for 18 successive generations and encountered no change in pathogenicity. Also, Christensen & Schneider (24) could not change the virulence of *H. savitum* by 10 successive passages through the host. This culture of *H. sativum* was derived from many successive monosporous isolations and it had grown on artificial nutrients for more than 28 years before it was passed back through the host. As the inoculations were made in each generation by means of single spores, chances of including a mutant were greatly minimized. Extensive population studies indicate that mutations in *H. sativum* occurred on the host in the ratio of 1:2900.

Changes in Virulence on Host

Changes due to chemotactic substances.—In 1904, Massee (58) concluded that a saprophytic fungus could gradually be educated to become an active parasite on a given host plant. When he grew *Trichothecium candidum* for 12 generations on leaves of Begonia injected with a 2 per cent sugar solution, the fungus acquired the faculty to attack normal leaves of this species. He also found that a parasitic fungus could be induced to attack a new unrelated host. When *Cercospora mëlonis* Cke. was grown for 21 generations on orchids which had been injected with an infusion from cucumber leaves, it became parasitic on the orchids. Also, as late as 1915, Massee (59) claimed that parasitism in fungi was an acquired habit, and Butler (15), in 1918, stated that certain saprophytes have become "educated" to a parasitic life. These results have never been verified and are not generally accepted.

In 1904, Salmon (82) concluded that a parasitic fungus may acquire the ability to attack an immune plant variety if the fungus is cultivated for a number of generations on an injured host and then transferred to an uninjured host of the same variety. By this means he claimed that powdery mildew of barley could be induced to grow normally on wheat. Although Hammarlund (45) grew powdery mildew of wheat for 125 asexual generations on injured barley, it never acquired the ability to attack uninjured barley. While on barley the organism did not lose its pathogenicity for the original host. Stakman (88) and others have been unable to substantiate Salmon's results.

Bridging hosts in rusts and mildews.—For many years there was considerable speculation on the influence of the physiologic condition of the host on the stability of rusts. Although Eriksson (31) demonstrated physi-

ologic specialization in rust fungi, he believed that rusts were gradually changed under the influence of climate and the host. Magnus (57) thought that strains of rust arose through the association of the fungus with the host-plant. Dietel (29) had similar views. Ward (99) made very extensive investigations with *Puccinia dispersa* Eriks. and concluded that the pathogenicity of races of brome rust could be changed through association with appropriate hosts. He also showed that whenever *P. dispersa* could not be transferred directly from one species of brome to another, the transfer could be accomplished by passing the rust through a third species, the bridging species, which usually was taxonomically intermediate between the resistant and susceptible host. Freeman (36) obtained similar results with this rust, and Freeman & Johnson (37) concluded that the *tritici* variety of *Puccinia graminis* could be induced to attack oats after it had passed through barley. Likewise, Johnson (52) was of the opinion that he could widen the host range of timothy rust, *P. phlei-pratensis* Eriks. & Henn. by using oats as a bridging species. Evans (32) concluded that hybrid wheats derived from crosses between resistant and susceptible varieties could act as bridging species and thus enable stem rust to attack the resistant parent. According to Salmon (82), *Erysiphe graminis* DC. also can be readily educated to attack immune plants by means of bridging hosts.

Alternate hosts also have been reported as bridging species for rusts. Arthur (1) thought that the barberry serves as a bridging host for stem rust and thereby increases its parasitic capabilities. Bolley & Pritchard (5) ascribed reinvigorating power to the barberry. At present it is well known that the sexual stage of stem rust occurs on the barberry and that new parasitic races of rust arise as a result of hybridization between biotypes on the barberry bushes.

Most of the successful experiments with bridging species were made long before it was recognized that the rusts and powdery mildew consist of many physiologic races and biotypes, and that new biotypes arise by means of hybridization, mutation, and heterocaryosis. This may help to explain the negative results obtained by several investigators who attempted to duplicate the early work of Ward, Salmon, and others. Whatever the cause, no one has been able to substantiate their results with more refined techniques. Stakman (88), Hammarlund (45), Stakman & Jensen (92), and Stakman & Piemeisel (90) found no indication of adaptation during long time association of parasites with the so-called bridging species.

There is fairly good evidence that rusts are remarkably stable. Stakman, Piemeisel & Levine (91) cultured *Puccinia graminis* for three consecutive years on barley and yet no new parasitic capabilities arose from this association. Later, Stakman, Levine, & Cotter (89) reported that uredinal cultures of *P. graminis tritici* race-1 had remained constant in pathogenicity for more than 13 years. Then when a change did occur, it was a sudden one, producing a mutant that differed strikingly in pathogenicity from its parent. Flor (34) carried four races of *Melamspori lini* (Schum.) Desm. for approxi-

mately four years and during this period they passed through 20 to 30 asexual generations and no changes occurred. According to Newton & Johnson (68) a number of mutations have been reported in several of the rusts and these genetic changes must be considered as a possible cause of what seems to be adaptation.

Adaptation of Phytophthora infestans.—*Phytophthora infestans* (Mont.) de Bary is cited frequently as a fungus that can readily be educated to attack resistant varieties of potatoes, That changes in pathogenicity occur is generally accepted, but the mechanism by which they occur is still controversial. Reddick & Mills (77) and Mills (61) were able to increase the virulence of *P. infestans* to a point where a number of normally immune varieties were severely blighted. Mills (61), by means of repeated passage of the potato strain through tomato was able to increase its virulence for that host. Once the pathogenicity was raised to a certain level, it remained there, even after 20 passages on any host. De Bruyn (13) obtained similar results using tomatoes. If increase in virulence is the result of educability, then the pathogen should also lose its acquired virulence by sojourn on appropriate hosts. Apparently this does not happen. In 1926, Berg (3) proved that there were races of *Phytophthora infestans* that differed greatly in parasitic abilities on potatoes and tomatoes. Later, Müller (67) recorded 31 races. Schick (84), Mills & Peterson (62) and others recognize races in *P. infestans*. If the fungus can be so easily molded by adaptation, the recognition of races can hardly be justified.

Investigations by Bonde, Stevenson & Clark (7) indicate that adaptation does not occur. They concluded that the virulence of *Phytophthora infestans* was not increased by propagation on resistant varieties in the field. This is in accord with the results of Müller (66, 67). Obviously, more exacting work is needed on this important problem.

Monosporous cultures.—Single spore cultures are not necessarily genetically pure cultures, as there is no assurance that mutation, segregation, or nuclear dissociation may not occur, even in a colonal line. Müller (65) passed three monosporous subcultures of *Gleosporium fructigenum* Berk. through certain hosts and materially increased their pathogenicity for their respective hosts. Changes that may occur in the host are well illustrated by studies made on *Helminthosporium gramineum.* (Rab.) Erik. Christensen & Graham (23) proved that two monoconidial lines of *H. gramineum* gave rise to variants while on a living host, whereas others did not. In one test, at least 10 distinct cultural lines, including parental lines, were isolated from a barley plant previously inoculated with a single monosporous line of *H. gramineum.* Monoconidial variants from this source again gave rise to new biotypes when passed back to barley. The exact method by which these variants arose was not determined. It is possible that the fungus was heterocaryotic, and this easily could account for the origin of these variants. Graham (44) found that anastomosing of hyphae was very common in *H. gramineum.* Shands & Dickson (85) obtained more than one race from a germinating conidium.

Mixed cultures.—Cultures may consist of similar cultural biotypes which differ greatly in virulence. Brierley (11) was not able to educate *Botrytis cinerea* Pers. except when the initial culture represented a mixed population. Cultures consisting of a mixture of races may be less virulent than the most virulent component. According to Blaug (9), the amount of wilt produced in a variety of flax inoculated with a mixture of two races of *Fusarium lini* Bolley was much less than the amount produced on the same variety inoculated with either of these races alone. Holton & Heald (51) obtained similar results with races of *Tilletia* spp. When a mixed culture is passed through a host, the weaker races may be eliminated and thus the pathogenicity of the culture may be increased. Under favorable conditions, a mixture of races may be perpetuated for several generations. Thus, Bailey (2) was able to perpetuate mixtures of races of *Cladosporium fulvum* for several generations both on nutrient media and on congenial hosts.

Experiments made by the senior author (20) demonstrated the ease with which apparent adaptation can take place when the initial inoculum consists of biotypes with different parasitic ability. In this test, two races of *Helminthosporium sativum*, one black and virulent, and the other an albino and weakly pathogenic, were mixed together in various proportions and inoculated on wheat and barley. There was a tendency for the albino race to be eliminated after several passages through the host, irrespective of the proportions of the albino used. When the albino inoculum was less than 50 per cent, it was seldom reisolated after the second passage of the mixture through the host. Also, the greater the proportions of the black used, the more severe the infection. The extent of "educability" was definitely associated with the genetic make-up of the particular components in the culture.

"Screening" or adaptation of smuts.—The influence of resistant hosts in the selection of new races of fungi is perhaps best illustrated in the smuts. It is well known that many new varieties of wheat, oats, and barley, resistant to their respective smuts, have lost their resistance after they have been grown commercially for a few years. These changes in varietal reaction are not associated with physiological changes of the host, but due to the development of new physiologic races of smut. How did they arise? By adaptation or by hybridization?

Dillon-Weston (30) and Bressman (10) obtained marked increase in percentages of bunt (*Tilletia* sp.,) on the formerly resistant variety Ridit, by reinoculation of this variety with bunt obtained from Ridit plants. Melchers (60) and Mourashkinsky (64) used a similar technique and obtained increased infection on other varieties. In a similar manner, Roemer & Kamlah (79) were able to increase the virulence of the loose smut *Ustilago tritici* Pers. of wheat. A number of workers [Reed (76), Nicolaisen (69, 70), Vaughan (97), Sampson & Western (83)] were able to increase the virulence of *U. avenae* (Pers.) Jens. and *U. kolleri* Wille when certain resistant varieties were reinoculated with the smut.

Conversely, Bever (4) was unable to obtain an increase in percentage of bunt infection when resistant varieties were reinoculated with their own smut for three successive years. Likewise, Sampson & Western (83) found that three of the six collections of oat smuts studied over a ten-year period did not change in pathogenicity. Vielwerth (98) obtained a decrease in virulence when the smut was propagated on a resistant variety.

Resistant varieties tend to select and increase the virulent biotypes from a mixture. Both Holton (50) and Bever (4) have presented evidence that the selective influence of the host is an important factor in the establishment of physiologic races of bunt fungi produced by hybridization. A collection of smut may be a mixture of two species of smut or a hybrid between them. Tervet (95) reported a case in which a collection of oat smut was originally identified as *Ustilago avenae*, but after several passages through Black Mesdag oats only *U. kolleri* could be isolated. The weaker species, *U. avenae*, apparently was screened out by the resistant oats. Flor (33) separated one race of *Tilletia caries* (DC.) Tul. from a mixed population of *T. caries* and *T. foetida* (Wallr.) Liro by the screening influence of several varieties of winter wheat.

Numerous experiments have demonstrated that collections of smut and even single smut chlamydospores may give rise to many biotypes. Therefore, changes in the virulence of smut are to be expected and are the result of selection of biotypes from chlamydospores which are heterozygous for factors governing pathogenicity. The chlamydospores represent the diploid phase of the smut and cannot be propagated independently. When they germinate, reduction and segregation of factors for pathogenicity occur; also, there must be fusion between compatible haploid lines before infection of the host can take place. Consequently, each parasitic generation may consist of a new group of biotypes. Therefore, the number of new races that can be "screened" by the host depends on degree of heterozygosity of original smut material.

CULTURAL ADAPTATION

Cultural adaptation has been reported with increasing frequency over the last half century. However, it is only in the yeasts that the phenomenon has been analyzed exhaustively, serving to elucidate basic relations between genes and the metabolism of microorganisms. The scientific contributions to this particular area are reviewed elsewhere in this volume (see Stanier, pp. 35–56). In contrast to the literature concerning adaptation in yeast, reports of superficially similar phenomena in other fungi are of a descriptive nature; consequently, this section will deal with work in the last ten years largely from the behavioristic viewpoint.

Adaptation, when restricted to nongenetic changes, does not cover cases such as those reported by Ryan (80), Bonner *et al.* (8), and Giles & Lederberg (40). Here, mutant strains of *Neurospora* requiring amino acids for growth were observed to revert to the wild type; it was established from

genetic data that reverse mutation to the wild type allele had occurred, a conclusion substantiated by an increased rate of reversion induced by x-rays (40).

The data of Ryan (80) and Ryan & Lederberg (81) illustrates, however, the difficulties involved in ascertaining whether reversion is due to selection or the formation of temporary enzyme systems. Reversion of leucineless *Neurospora* strains is variable with respect to frequency, time required, and degree. It is more frequent at low concentrations of added leucine and with small amounts of inoculum, a result not expected if reversion results from spontaneous back mutation. Heterocaryons composed of wild-type (L) and mutant (l) nuclei grow at the wild type rate in the absence of leucine, but at the mutant rate with added leucine, indicating that l nuclei dominate L nuclei through inhibition or competition in the presence of leucine. Consequently, the frequency of reversion in this instance will be governed by (*a*) the rate of back mutation to the wild type allele, (*b*) the degree of dominance, and (*c*) the probability that L nuclei will become isolated from the dominant mutant nucleus. The latter two factors will be determined by the ratio of l to L nuclei and by the concentration of leucine in the medium.

The complexities found in the analysis of selection in *Neurospora*, where genetic data are considerably more abundant than in other fungi, emphasize the necessity for caution before adaptive processes of a temporary nature may be considered established for fungi whose nuclear condition and mutability are imperfectly known.

Adaptation to carbohydrates.—With the increasing utilization of fungi in a variety of industrial applications, their ability to attack a large number of diverse substrates is being increasingly exploited. Nordstrom & Hultin (72) obtained preparations of enzymes, previously not recorded, by placing thirty species of molds in contact with dextran, a glucose polymer containing alpha-1,6 linkages. *Penicillium lilacinum* Thom. *P. funiculosum* Thom, and *Verticillium coccorum* (Petch) Westerdijk were able to hydrolyze the polymer after exposure for one week. Amylase and saccharase were not produced in media containing dextran, while on starch only amylase was elaborated. A similar procedure was followed by Gottlieb, Day & Pelczar (28, 42) in obtaining cultures of *Polyporus abietinus* (Dicks.) Fries and *Poria subacida* Pk. (Sacc.) able to utilize lignin as an energy source. Originally grown in a mixture of lignin and glucose, successive transfers to media increasingly dilute with respect to glucose eventually resulted in cultures dissimilating lignin. Foster *et al.* (35) found that *Penicillium chrysogenum* Thom grown previously on glucose required 50 hr. to ferment lactose, while lactose-grown cells accomplished the fermentation in 26 hr. Lactose was a more efficient carbohydrate source for penicillin production. Nielsen & Nilsson (71) have demonstrated the practicality of xylose as a substrate for fat formation in *Rhodotorula gracilis* Renn. Initially, xylose was fermented at 25 per cent of the rate of glucose, but continuous transfer to xylose increased the rate to 70 per cent. The economic coefficient (gram cells formed per gram sugar

utilized) eventually attained the same value for both sugars. Gould & Tytell (43) found that *Fusarium* (probably *tricothecoides*) grown on glucose would not ferment galactose, xylose, arabinose, lactose, or maltose. Cultured on galactose, the organism subsequently fermented the compounds as indicated by an R. Q. greater than 1. According to Wasserman & Gould (100), *Neurospora crassa* Shear and Dodge, lysineless strain 4545, ferments glucose more rapidly than it does fructose, but fails to produce saccharase in the presence of glucose. Sugars yielding glucose also inhibit saccharase production, while fructose or derivatives enhance production. Goodman (41) has followed the changes in amylase production in fungi responsible for the deterioration of stored grain. Mycelial preparations of *Aspergillus flavus* Link., *A. niger* Van Tieghem, and *Penicillium notatum* Westling did not possess amylase activity if grown on glucose, but on starch the content was high at the end of five days. Subsequent loss of activity may be attributable to dilution of the enzyme by inert cell wall material. Exocellular production appeared to be limited to a period between five and eleven days. Similar results were obtained with lipase.

Weimer & Harter (101) were among the first to investigate the possibility of adaptive behavior in the enzyme complexes associated with pathogenicity for higher plants. Gäumann & Bohni (38, 39) and Pfaff (74) have further investigated adaptation to pectin, but unfortunately some confusion is created by the use of different systems of enzymatic nomenclature. *Botrytis cinerea* Pers. (38) and *A. niger* Van Tieghem (39) both possess an exocellular polygalacturonidase which is constitutive, reaching maximum activity independently of the mycelial mass of the culture. On the other hand, pectase is present only when pectin is available and increases concomitantly with mycelial weight. Pfaff (74) obtained similar results but believed that the enzyme responsible for degration of the galacturonic chain was also adaptive, since only molecules possessing the galacturonic acid structure elicited formation of the pectin enzyme complex.

None of the data reported above show that response to the substrate is due to the formation of temporary enzyme stystems. Rather, the probability of selection of variants is good, since in all instances the cells were proliferating and no attempts to obtain immediate reversion to the original condition were made.

Adaptation for nutritional requirements.—The back reversion of mutants of *Neurospora* to wild type strains capable of synthesizing all compounds except biotin has already been cited as a case of selection, but there are many instances where this explanation cannot be applied. Tatum & Beadle (94) found the growth of a *p*-aminobenzoic acid (PAB) requiring mutant to be slightly but significantly stimulated by pimelic acid, although the effect was not noticeable until five days after exposure, in contrast to the one day necessary for response to PAB. Recovering and retesting these strains indicated that a genetic change was not involved. Regnery (78) has reported a leucineless strain for which leucic acid alone does not suffice, but which

will utilize this compound if sub-optimal concentrations of leucine are present. Srb & Horowitz (87) observed incidentally that wild type *Neurospora* converted 14 per cent of added arginine to urea and ornithine if grown on low levels of arginine, but only 6 per cent was converted if grown on minimal media. In the first two instances, failure of succeeding conidial transfers to perform the acquired function might be construed as evidence for a temporary adaptive change. But in view of the influence of substrate on the interactions between nuclei, it would be necessary to test a large number of monosporous isolates to insure absence of heterocaryosis in these responses.

Adaptation to toxic materials.—Microbiologists often are amazed at the variety of unusual conditions tolerated by fungi. Sletten & Skinner (86) found a species of *Trichosporon* attaining considerable growth in sulfuric acid solutions up to 2.5 *N*. The same isolate was capable of appreciable development in 1 *N* sulfuric acid supplemented with glucose, peptone, and 280 gm. of copper sulfate per liter.

A gradual increase in tolerance to copper sulfate is reported by Mader & Schneider (56). Five monoascosporous isolates of *Sclerotinia fructicola* (Wint.) Rehm grew well in the presence of 10,000 p.p.m. of copper sulfate after 18 weeks exposure to 5,000 p.p.m. although the cultures initially failed to grow at 7,000 p.p.m. When transferred back to ordinary potato-dextrose agar (PDA), some cultures reverted rapidly to the original type in morphology and copper tolerance, while others were mutants in both respects. Determination of the occurrence of mutation by comparison on PDA is not rigorous, since Mader & Teller (55) have shown that cultures appearing identical on neutral PDA can be separated as distinct biotypes simply by lowering the pH. Hirt (48) has reported a case of acquired tolerance to copper by *Poria xantha* Lind., but the associated morphological alterations strongly indicate the selection of a copper tolerant variant.

Haploid, mutable and nonmutable, lines of *Ustilago zeae* (Beckman) Unger increased their tolerance for sodium arsenite from 2,400 p.p.m. to 7,000 p.p.m. during 10 transfer generations on gradually rising levels of arsenic [Stakman *et al.* (93)]. At the end of 4 transfer generations on normal PDA, they appeared to have reverted to the original lines. Because of the difficulty in recognizing morphologic mutants on toxic media, Hirschorn & Munnecke (47) attempted to cross the same adapted and nonadapted lines to observe if segregation occurred for arsenic tolerance, but were unsuccessful because of loss of combining ability which accompanied the increase in tolerance. Loss of tolerance resulted in gains in combining ability. Wilson (103) has adapted two species of *Sclerotium* to arsenic in a similar manner.

Investigation of the mechanism of acquired tolerance to toxic materials is hampered by the radical changes in appearance that most organisms experience under these conditions, thereby making identification of sectors difficult. Christensen (19), however, found that increased tolerance of *Gibberella zeae* to mercury bichloride, ethyl mercury phosphate, and mala-

chite green could be explained by mutation as determined from comparison of cultural characters on a variety of media, by changes in pathogenicity and failure to revert to susceptible types. Even if selection is considered unlikely, the demonstration of adaptive enzymes is rendered difficult because of the lack of a clear-cut relationship between an enzyme and substrate in these instances. A specific relationship between arsenic and the formation of organo-arsenic compounds of the type described by Challenger (17) is feasible, but it is possible that adaptation to inorganic material may arise through general detoxification mechanisms which are unknown at present.

Phenotypic variability.—In addition to acquired ability to utilize organic substrates or tolerate toxic agents, fungi exhibit other cultural behaviors which resemble adaptation in some respects. Pronounced temporary modifications of morphology and pigmentation in a single isolate are produced commonly as a consequence of growth on differential media. In most cases it is impossible to characterize the substances initiating the change because of the complex composition of plant or animal extracts provided as substrates. Variability is not confined to changes in cultural appearance, but is a feature of considerable importance in industrial mycology and plant pathology.

Studies of the influence of synthetic media on rates of metabolism and nature of metabolic pathways may explain in some degree the reasons underlying phenotypic variability, suggesting possibilities for investigations of more complex media. Hofmann, Scheck & Saffert (49) found that the growth of *Aspergillus oryzae* (Ahlburg) Cohn was maximum in solutions containing 400 μg. of manganese, but that saccharase production was at a maximum with no added manganese. The reverse was true for catalase. In addition, β-glucosidase activity varied depending on whether Raulin's or Nikla's culture medium was employed, although growth was essentially the same on both media. According to Ozawa & Takeda, the elaboration of exocellular protopectinase (73) by *A. oryzae* (Ahlburg) Cohn and *Gibberella saubinetti* (Dur. and Mont.) Sacc. varies according to carbon source, appearing in the culture medium if starch, dextrose, or pectin are substrates, but not other sugars. Potassium sulfate and potassium nitrate accelerate the secretion of protopectinase, but potassium sulfate does not promote growth. These results are not in conformity with those obtained by Gäumann & Bohni (39) with *A. niger*, but the differences might be due to species differences.

Responses of the type just described are not equivalent to the formation of adaptive enzymes because the increased rate of activity does not arise through the presence of the homologous substrate for the enzyme. However, the gross similarity between the two processes may justify continued examination of phenotypic variability in this manner.

CONCLUSION

The above summary shows the need for caution in generalizing with regard to the nature of changes in virulence and cultural characteristics of

fungi. In general, the mass of evidence in this field indicates that fungi do not change their pathogenicity as a result of gradual adaptation, but when changes do occur they can usually be explained on the basis of genetic changes: mutation, hybridization, and nuclear dissociation or reassociation (heterocaryosis). Most of the workers give their results without attempting to analyze genetically their final population. In many cases, the genetic purity of material used may be questioned. In some instances the original isolates were single biotypes and in others two or more biotypes were definitely present. Sometimes the results were based on data too meager to prove or to disprove the adaptation or bridging phenomenon, and in many instances there were misinterpretations of the experimental data because of unfamiliarity with the genetic variation of the fungus. These same general considerations apply to changes in physiological function on artificial media or in the presence of toxic materials, except that in most cases no genetical analyses have been made. In these instances, the lack of data on genetic behavior leaves the question of adaptation a matter for speculation.

Much of the evidence presented indicates that the potentialities of a fungus species, like those of higher plants, are limited by their genes. Evidently many more exacting studies are required in this field before the extent of plastic departure beyond limitations imposed by the genes can be definitely stated.

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METABOLISM OF BACTERIA

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This review covers approximately the period from January to December, 1950. Due to the ever-increasing flood of publications, all papers in the field could not be analyzed. A choice has been made, based upon the interests of the reviewer, and the fact that other reviews in this issue (1, 2) deal with more specialized aspects of the metabolism and nutrition of bacteria. The reviewer begs to apologize in advance for any omission of important papers which may have resulted from the fact that the latest issues of certain journals were not available in France at the time of writing.

Carbohydrate Metabolism

A preliminary report by Caputto *et al.* (3) mentioned that a thermostable factor is necessary for the enzymatic conversion by extracts of *Saccharomyces fragilis* of galactose-1-phosphate to glucose-1-phosphate. This factor has now been obtained in almost pure form by the same workers and its constitution has been established. It contains uridine, two phosphate groups, and glucose, and has been named uridine diphosphate glucose. A tentative formula is given in which glucose is linked through carbon 1 to the terminal phosphate group in uridine-5′-diphosphate. Methods are given for its isolation from baker's yeast and for its estimation (4, 5).

Pappenheimer & Shaskan (6) had previously shown that in iron-deficient media, the normal fermentation of *Clostridium perfringens* is diverted to a homolactic fermentation, which still requires iron. Aldolase, the enzyme converting hexose diphosphate to triose phosphate, has been obtained in a cell-free form from *C. perfringens* by Bard & Gunsalus (7) and has been shown to be inhibited by the same chelating agents that inhibit glycolysis. The inhibitions of both the homolactic fermentation and the aldolase reaction are reversed by ferrous, or less completely by cobaltous ions. The presence of aldolase in this organism suggests the occurrence of the Embden-Meyerhof system, a point previously questioned. Furthermore, the paper mentions that the aldolase-containing extracts of *C. perfringens* contain also an isomerase and an ethanol dehydrogenase.

Norris & Campbell (8) had previously established that gluconic and 2-ketogluconic acids are intermediates in the oxidation of glucose by *Pseudomonas aeruginosa*. They find now (9) that neither the fermentation liquor nor the cells of this organism contain any of the hexose members of the Embden-Meyerhof scheme. They conclude that gluconic and 2-ketogluconic acids are on the main path of glucose dissimilation by this organism and other paths do not exist. Further evidence lies in the fact that a pentose

phosphate may be formed and that only one mole of acetic acid is formed per mole of glucose oxidized (10).

Koepsell (11) described conditions where *Pseudomonas fluorescens* grown in synthetic medium oxidizes gluconate with accumulation of pyruvate and α-ketoglutarate up to high levels. Gluconate oxidation is markedly stimulated by traces of iron. The data given and the presence of 2-ketogluconate among the dissimilation products suggest that the latter is an intermediate in this fermentation.

Cohen & McNair Scott (12) extracted from *Escherichia coli* adapted to gluconate an enzyme, gluconokinase, which catalyzes the reaction

gluconate+adenosinetriphosphate→6-phosphogluconate+adenosinediphosphate. (A)

They could not obtain evidence for the existence of this enzyme in the unadapted cells. When *E. coli* is adapted to gluconate, it is not simultaneously adapted to D-arabinose, arabonate, 2-ketogluconate, and 5-ketogluconate. The adaptation thus seems to involve only the formation of gluconokinase. Phosphogluconate is degraded exclusively via the oxidative pathway through pentose phosphate.

The effect of potassium depletion has been studied by Roberts & Roberts (13) in *E. coli*. Potassium does not appear to be necessary for the oxidation of hexose phosphates through pentose phosphates, but is required in the anaerobic reaction sequence.

Wild-type *Pseudomonas putrefaciens* cannot grow with glucose as carbon source or utilize it at an appreciable rate. A mutant has been obtained by Klein & Doudoroff (14) that can both utilize glucose as a carbon source for growth and oxidize it rapidly. The utilization of glucose by the mutant is strictly adaptive, the phenotypic difference lying in the capacity of the mutant to produce a glucose-specific hexokinase, whereas the wild-type can not. Both strains have all the other enzymes necessary for the further metabolism of glucose-6-phosphate.

The β-D-galactosidase of *E. coli* has been extracted by Lederberg (15) from adapted cells of strain K-12. The method of assay is based upon the colorimetric determination of *o*-nitrophenol released from the chromogenic substrate *o*-nitrophenyl-β-D-galactoside (ONPG), which was prepared by Seidman & Link (16). The kinetics of hydrolysis of this substrate agree with the Michaelis theory. The action of various ions on the activity of the enzyme has been studied. With activity in dilute potassium phosphate buffer as reference, lithium, ammonium, and caesium are inactive. Sodium stimulates, while rubidium is inhibitory. Rubidium inhibition is reversed by sodium or potassium. Galactose and galactosides other than ONPG, such as lactose, combine with the enzyme and behave as competitive inhibitors of ONPG hydrolysis. The apparent galactosidase activity of intact cells is enormously increased by drying or autolysis. Small amounts of the enzyme are found in unadapted cells.

Monod *et al.* (17) and Monod (18, 19) had previously described the

isolation, the partial purification, and some properties of the same enzyme which had first been called "lactase." Cohn & Monod (20) have done considerable work on this enzyme; they have described the purification of the β-galactosidase from *E. coli mutabile* adapted to lactose. The cells are extracted by treatment with toluene in distilled water. After elimination of the bacterial bodies, the supernatant contains 40 to 70 per cent of the total original activity. The enzyme is then purified by ammonium sulfate fractionation. The preparation hydrolyzes lactose and ONPG quantitatively and is inactive on all compounds that do not have the β-galactosidic structure. The following facts show that lactose and ONPG are hydrolyzed by the same enzyme: (*a*) The rate of thermal inactivation is the same for the hydrolysis of lactose and ONPG. (*b*) Electrophoresis shows the presence in the preparation of at least two constituents, both of which are active on lactose and ONPG. The ratio of the activities towards the substrates is the same for the two fractions. (*c*) An anti-lactase serum has been obtained by immunization of rabbits with the purified preparation. The quantitative study of the precipitation reaction indicates that the activity towards lactose or ONPG is associated with a single antigen. The anti-galactosidase serum precipitates also with the lactase of *Aerobacter aerogenes*, *E. coli* K-12, but not with that of *Saccharomyces fragilis*. Cohn & Monod (20) have also investigated the influence of monovalent and bivalent cations on the enzymatic hydrolysis of lactose. Most monovalent cations are found to be activating, when used alone. However, sodium may exert an inhibitory or an activating effect, depending on the presence of other ions. These "pseudo-antagonistic effects," as well as the inhibitory effects of certain ions such as lithium, could be understood as resulting from competitive relationships between ions endowed with different affinities and different powers of activation for the enzyme. A new constant called "activance" is introduced, defined as the activity of the enzyme, when saturated by the ion. When lactose is the substrate of β-galactosidase, potassium is the ion with the highest "activance"; on the contrary, when ONPG is the substrate, sodium "activance" is higher than that of potassium. This remarkable observation demonstrates that "activance" depends upon the chemical structure of the substrate.

Cohn & Torriani (21) observed that the anti-galactosidase serum, which precipitates totally the enzyme (G_z), does not inhibit its activity, which is found entirely in the precipitate. They found that extracts of unadapted *E. coli* which possess almost no activity, contain an antigen (P_z) which precipitates almost totally with the anti-galactosidase. They have been able to titrate simultaneously G_z and P_z and made the interesting observation that crude extracts of unadapted cells contain only P_z, while extracts of the adapted cells contain both the antigens G_z and P_z. These results demonstrate that the adaptive formation of β-galactosidase involves the synthesis of a new antigen and that a protein exists in the unadapted cells which has a structure very close to that of the enzyme molecule. The adaptation, which

leads to a synthesis of G_z is specifically accompanied by a decrease in the amount of P_z, which appears as a possible precursor of the enzyme.

Monod & Torriani (22) continued their work on amylomaltase from *E. coli*. The enzyme has been obtained cell-free and further purified by ammonium sulfate fractionation and dialysis; it seems specific for maltose. The enzyme is a transglucosidase and the reaction catalyzed is

$$n\ \alpha\text{-glucosido-4-glucose} \rightleftarrows (\text{glucose})^n + n\ \text{glucose} \qquad \text{(B)}$$

This reaction is reversible: in the presence of glucose, the polysaccharide is degraded and maltose is formed.

Spiegelman *et al.* (23) found that in *Saccharomyces cerevisiae*, adaptation to maltose leads to the production of two enzymes, one capable of splitting maltose and α-phenylglucoside, the other inactive towards these substrates, but very active against α-methylglucoside. No evidence of the presence of an amylomaltase-type enzyme was found.

The maltase of *Clostridium acetobutylicum* has been separated from amylase by French & Knapp (24). It is not specific for maltose as previously stated (25), but also hydrolyzes at an appreciable rate starch, dextrins, and some oligosaccharides.

Aubel *et al.* (26 to 30) extensively studied the dissimilation of glucose by nonproliferating *E. coli* under aerobic and anaerobic conditions. They studied the action of fluoride, malonate, and azide on the phosphorylation accompanying respiration as well as fermentation (27, 29). They emphasized the importance of the permeability of the cell membrane in the study of bacterial metabolism: thus, fluoride does not penetrate the intact *E. coli* cells in the presence of glucose; the authors observed that the addition of succinate makes this penetration possible (30). The general conclusion of these investigations is that the respiration of *E. coli* can not be defined as a series of processes leading to the fixation of hydrogen on active oxygen by means of the Warburg-Keilin system, but rather as a fermentative process where oxygen acts merely as an unspecific hydrogen acceptor.

Fowler (31, 32) observed that when *E. coli*, growing exponentially in air, is transferred to anaerobiosis, there is an immediate cessation of growth. After 30 to 50 min., the growth resumes at an increasing rate until the anaerobic growth rate is about the same as the growth rate in air. There is no measurable uptake of glucose during the period of no growth. The utilization of glucose starts just before visible growth and rises abruptly to a maximum of at least 2.5 times the aerobic rate. The evolution of carbon dioxide increases at least ten-fold during this same period. The increase in fermentation can be completely inhibited with 2,4-dinitrophenol if it is added before the fermentation has reached the maximum rate. Infection with bacteriophage also inhibits any increase in fermentation. The results are interpreted to mean that the anaerobic utilization of glucose is effected by an adaptive enzyme system. Dinitrophenol (DNP) (33) and bacteriophage (34) are known to inhibit enzymatic adaptation. The supposed adap-

tive enzyme is much more labile than any so far reported, since it de-adapts rapidly in the absence of an exogenous nitrogen source and in the presence of air. DNP protects against these losses of activity. The dual effect of DNP in protecting against both the increase and decrease of adaptive activity was first shown by Spiegelman *et al.* (35). The results suggest that air inhibits, not the functioning, but the synthesis of some enzyme or enzymes in a strictly fermentative system and may offer a new interpretation of the Pasteur effect.

Ephrussi & Slonimski (36) found that if bakers' yeast is grown in anaerobiosis, it cannot utilize glucose by respiratory mechanisms; it loses at the same time cytochromes-*a*, *b* and *c* and acquires two new components with the properties of cytochromes-a_1 and b_1. Oxygen induces in such a yeast the adaptive synthesis of normal cytochromes in the absence of cell multiplication, provided that an exogenous source of energy is supplied. Simultaneously, the two new components disappear and the organism again becomes able to respire. Slonimski (37) showed that if a mutant of this yeast, genotypically unable to respire, and containing only cytochrome-*c* is grown anaerobically, cytochrome-*c* is lost and the supposed precursors a_1 and b_1 appear; during adaptation in air, cytochrome-*c* is synthesized, but remains always in the reduced state. This is an example of adaptive synthesis of a substance which is apparently of no use to the organism.

Assimilation.—Siegel & Clifton (38) presented evidence which indicates that energy production and substrate assimilation are not equivalent criteria in predicting the development of *E. coli* cells; for example, lactose is assimilated to a lesser extent during growth than arabinose or glucose, though a smaller yield of energy is derived from respiration during growth on arabinose. The same authors (39) observed that the efficiency of assimilation of different substrates by the growing cell is generally independent of the free energy of oxidation of the substrate, since a greater degree of assimilation was noted with fumarate than with succinate, and with pyruvate than with lactate.

Oxidation.—O'Kane (40) found that a particular strain of *Streptococcus faecalis* rapidly oxidizes glucose in the presence of the pyruvate oxidation factor [O'Kane & Gunsalus (41)]. Under the conditions used acetate and carbon dioxide are the main products of glucose oxidation. In the absence of the factor, pyruvate accumulates or is removed by acetylmethylcarbinol formation. The data indicate that diphosphopyridine nucleotide (DPN) and a flavin derivative are implicated in the respiration of the organism under study.

VanDemark (42) showed that thiamine, nicotinic acid, riboflavin and an unidentified factor are concerned as coenzymes in the oxidation of glycerol by *S. faecalis*. The unknown factor is apparently not a common amino acid, mineral, or known growth factor. The pyruvate oxidation factor of O'Kane & Gunsalus was not tested by the author.

Haugaard (43) demonstrated the presence in *E. coli* of two enzyme systems for the oxidation of lactic acid, one oxidizing D-lactic acid, the other

L-lactic acid. The system oxidizing lactate is associated with particulate matter. Acetone treatment of the suspension obtained by ultrasonic vibration destroys specifically the system oxidizing L-lactic acid.

Lindsay, O'Donnell & Edson (44) showed that *Mycobacterium ranae* suspensions oxidize pyruvate and acetate at equal rates. Normal fatty acids are oxidized faster. In the presence of iodoacetate, pyruvate is quantitatively converted to acetate, the metabolism of which is then suppressed. Ajl, Hart & Werkman (45) found that when biotin is added to a dialyzed cell-free extract of *E. coli* in the presence of α-ketoglutaric acid, an increase in oxygen uptake is observed. This increase is lower when malonate is added. It has been found that biotin functions in the oxidation of succinate and that the increase in the oxidation of this metabolite is not due to the oxidation of oxaloacetate arising from succinate. Fumarate is not appreciably attacked by the extracts either with or without biotin; carbon dioxide is not formed from succinate; the oxidation of L-malate or acetate is not increased by biotin. Randles (46) brought evidence for the probable oxidation of fatty acids by *Neisseria catarrhalis* through β-oxidation.

Quastel *et al.* (47) showed that two species of *Achromobacter* and one of *Corynebacterium* will oxidize pyruvic oxime to nitrite. The pyruvic oxime oxidase has the characteristics of an adaptive enzyme. The ratio moles of oxygen consumed per mole of nitrite formed is 1.78. This indicates that oxidation of pyruvate occurs also. Other oximes, which are not oxidized, are inhibitory. The oxidation of pyruvic oxime by *Achromobacter* is poisoned by cyanide and azide. Franzl & Chargaff (48) obtained from *Acetobacter suboxydans* a cell-free enzyme preparation which will oxidize *meso*-inositol with the utilization of one atom of oxygen per mole of inositol. Calcium and magnesium enhance the activity of the preparation. Richtmeyer *et al.* (49) showed that *A. suboxydans* oxidizes L-fucitol (6-desoxy-L-galactitol) to the new sugar L-fuco-4-ketose.

Stanier *et al.* (50) found that cells of *Pseudomonas fluorescens*, grown on mandelate, benzoate, or phenol, and then dried, oxidize catechol and protocatechuic acid to β-ketoadipic acid with an oxygen uptake of one mole per mole of substrate and the evolution of one mole of carbon dioxide. The mechanism of the enzymatically catalyzed oxidation of protocatechuic acid to β-ketoadipic acid has been investigated further by Stanier (51), who has obtained the enzyme cell-free by aqueous extraction of dried cells of *P. fluorescens*. When dialyzed, the enzyme lost the ability to produce carbon dioxide, but retained the ability to oxidize protocatechuic acid with the original uptake of oxygen. This indicates that the intermediate formed from protocatechuic acid by the dialyzed preparation is a 7-carbon tricarboxylic acid, which can undergo a non-oxidative decarboxylation to β-ketoadipic acid when exposed to the complete enzyme system. Karlsson (51a) studied a mutant of *Azotobacter agilis*, isolated as a variant unable to grow on glucose. This strain is completely unable to develop on compounds which are utilized for growth by the parent strain such as pyruvate, lactate,

malate, fumarate and succinate. Growth of the mutant is supported only by ethyl alcohol, acetate, and malonate. Certain compounds which do not support growth of the mutant are, however, oxidized by it. The behavior of the mutant can be accounted for by the assumption that it has a metabolic block in the conversion of oxaloacetate and pyruvate to acetate. The latter is postulated to be indispensable for the growth of *A. agilis*.

Acetate metabolism.—Acetyl phosphate is not always the active form of acetate. Lipmann (52) found that it is inactive as an acetyl donor for the acetylation of sulfanilamide by pigeon liver extracts. Kaplan & Lipmann (53) have shown that *E. coli* extracts contain an enzyme which transforms acetate plus ATP into "active acetate." The partially purified barium salt of this compound could not be differentiated chemically from synthetic acetyl phosphate but appears to be more resistant to the action of muscle acetylphosphatase.

Chou, Novelli, Stadtman & Lipmann (54) found that extracts of *E. coli*, *Clostridium butylicum*, and *C. kluyveri* contain an enzyme capable of transforming acetyl phosphate into an active acetyl donor for citrate or acetoacetate synthesis by acetyl acceptor fractions of pigeon liver extracts. Stern & Ochoa (55) also find that *E. coli* and *A. agilis* extracts form "active acetate" from acetate plus ATP. The enzyme involved is absent from heart, kidney, brain, and other tissues, but if a small amount of the microbial enzyme is added to these systems, important quantities of citrate are formed from oxaloacetate plus acetate plus ATP.

It has been shown recently by Stern & Ochoa (56) that a pigeon liver extract is capable of condensing acetate and oxaloacetate to citrate, provided that magnesium or manganese ions, ATP, and coenzyme A are present. Novelli & Lipmann (57) have now extended this finding to cell-free extracts of yeast and *E. coli*. In extracts of *E. coli*, synthetic acetyl phosphate may act as acetyl precursor in citric acid synthesis preferentially to acetate plus ATP. Stern & Ochoa (55) find similar results with extracts of *A. agilis*. This last observation seems in contradiction to a previous report by Karlsson & Barker (58), who concluded that acetate is not oxidized by this organism via the tricarboxylic acid cycle since the utilization of acetate doubly labeled with C^{14} does not give rise to any radioactivity in succinate or oxaloacetate under conditions where these compounds accumulate.

The fact that citrate rather than isocitrate or *cis*-aconitate is formed is in agreement with the theory of Ogston (59) that citric acid can act like an asymmetric molecule in an enzyme-substrate complex. This concept has been substantiated by Potter & Heidelberger (60, 61) and explains how "symmetrical" labeled citric acid can lead to an asymmetrical distribution of isotope in the carboxyl groups of α-ketoglutaric acid.

Stadtman and Barker (209) found that extracts of *C. kluyveri* contain a transacetylation enzyme, which Stadtman (62) considerably purified by ammonium sulfate fractionation. The enzyme is inactivated after several refractionations; its activity is restored upon addition of coenzyme A. This

purified fraction renders synthetic acetyl phosphate available as an acetyl donor to coenzyme A-dependent liver acetyl-acceptor systems (citrate, acetoacetate, and acetylsulfanilamide synthesis). The preparation is devoid of the enzyme transforming acetate plus ATP into "active acetate." The same preparation catalyzes the interchange of acetyl-bound phosphate with inorganic phosphate.

Stern, Shapiro & Ochoa (63) extended their studies and found that at least two enzymes are required for citrate synthesis in *E. coli* extracts with acetyl phosphate and oxaloacetate as substrates: (*a*) an enzyme activating acetyl phosphate; (*b*) another enzyme which catalyzes the condensation of "active acetate" with oxaloacetate to citrate. The over-all reaction is:

$$\text{acetyl phosphate} + \text{oxaloacetate} \rightarrow \text{citrate} + H_3PO_4. \qquad \text{(C)}$$

Enzyme *a* is thought to be identical with the transacetylase obtained by Stadtman (62) from *C. kluyveri* extracts; it has been obtained from *E. coli* and *C. butylicum* practically free of enzyme *b*. Enzyme *b* is readily soluble and is present in various animal tissues, in *E. coli*, *A. agilis*, *Mycobacterium tuberculosis*, and yeast. It has been obtained in crystalline form by Ochoa, Stern & Schneider (64) from pig heart; it catalyzes the synthesis of citrate from acetyl phosphate and oxaloacetate, provided that coenzyme A and a source of enzyme *a* (*E. coli* extract for example) are present. The condensing enzyme catalyzes also a slow exchange between radioactive acetyl-bound oxaloacetate (*i.e.*, citrate) and free oxaloacetate in the presence of coenzyme A. This indicates a reversibility of reaction (C); however, the equilibrium is far in favor of citrate synthesis. In this last experiment, the radioactive citrate was prepared by the use of *Aspergillus niger* fermenting glucose in the presence of $C^{14}O_2$. All the radioactivity of the citrate was equally distributed in the two carboxyls of its oxaloacetate moiety. A similar study of citric acid formation by *A. niger* in the presence of $C^{14}O_2$ has been done by Martin, Wilson & Burris (65).

Following the work on citrate synthesis from acetyl phosphate plus oxaloacetate, Korkes, Stern, Gunsalus & Ochoa (66) studied citrate synthesis from pyruvate plus oxaloacetate by microbial extracts. In the presence of orthophosphate, soluble enzyme preparations of *E. coli* and *Streptococcus faecalis* catalyze the dismutation of two molecules of pyruvate to acetyl phosphate, lactate and carbon dioxide. For full activity the system requires diphosphopyridine nucleotide (DPN), magnesium or manganese ions, cocarboxylase, lactic dehydrogenase, and a boiled extract of yeast. Coenzyme A is required for maximum yields of acetyl phosphate, but is not the active factor of yeast extract, which is possibly a coenzyme-bound form of the pyruvate oxidation factor of O'Kane & Gunsalus (41). The dismutation reaction is formulated as follows:

$$\text{pyruvate} + \text{phosphate} + DPN_{ox} \rightarrow \text{acetyl phosphate} + CO_2 + DPN_{red}. \qquad \text{(D)}$$

$$\text{pyruvate} + DPN_{red} \rightarrow \text{lactate} + DPN_{ox}. \qquad \text{(E)}$$

If phosphate is replaced by oxaloacetate plus condensing enzyme *b* (which is absent from the extracts utilized), no acetyl phosphate is formed and citrate is synthesized instead. The authors explain their results by assuming that reaction (D) may result in the formation of an acetyl-enzyme or acetyl-coenzyme intermediate which may be cleaved in the presence of another enzyme either by phosphate with formation of acetyl phosphate or by oxaloacetate with formation of citrate; this would regenerate the hypothetical coenzyme, which may well be coenzyme A. This explanation is supported by the fact that citrate synthesis occurs in the almost complete absence of orthophosphate. Phosphate actually slightly inhibits citrate synthesis.

This recent information on the activation of acetate and acetyl phosphate by microbial enzymes explains the activity of acetyl phosphate in citrate synthesis by microorganisms [Novelli & Lipmann (57); Stern, *et al.* (55, 63)] and in butyrate synthesis by *C. kluyveri* [Stadtman & Barker (67)]. It may even be concluded, as Potter & Heidelberger (68) point out "that most if not all acetate reactions are mediated by coenzyme A, which functions as an acetate acceptor-donor system with various specific enzymes, each catalyzing a different acetate donating or accepting reaction. In other words, active acetate appears to be a complex of coenzyme A, enzyme and C_2-fragment."

However, a point remained unexplained. Strecker, Wood & Krampitz (69) found that extracts of *E. coli* which rapidly fix formate in the carboxyl group of pyruvate do not fix either synthetic acetyl phosphate or an acid anhydride formed from pyruvate. Furthermore, *Staphylococcus aureus*, which shows no phosphoroclastic reaction similar to that of *E. coli*, does fix formate in pyruvate. They conclude that acetyl phosphate as such is not involved in the fixation of formate in pyruvate and consider other possibilities for explaining their results.

Chantrenne & Lipmann (70) offered an explanation for these facts. They found that the exchange of formate with the carboxyl group of pyruvate in extracts of the strain of *E. coli* utilized by Strecker *et al.* (69) is a coenzyme A-dependent reaction; if CoA is removed by treatment with an anion exchange resin, the extracts become inactive; and activity is restored upon addition of CoA. The authors propose that in this reaction an acetyl-CoA-enzyme complex is formed intermediately which can serve as acetyl donor. Through this system, pyruvate can act as an immediate acetyl donor without intermediate formation of either acetate or acetyl phosphate. According to these views, the phosphoroclastic splitting of pyruvate in *E. coli* should be reformulated as follows:

$$\text{pyruvate } (=\text{acetyl}\sim\text{formate}) + \text{CoA-enzyme} \rightleftarrows \text{acetyl}\sim\text{CoA-enzyme} + \text{formate.} \quad \text{(F)}$$

Ajl (71) obtained evidence which indicates that the citric acid cycle does not occur in the respiration of *E. coli* and *Aerobacter aerogenes:* pyruvate is oxidatively converted to carbon dioxide and acetate; the simultaneous adaptation technique indicates that acetate is not oxidized via *cis*- aconitate

and α-ketoglutarate, but rather through succinate and the Szent-Györgyi dicarboxylic acid system, with the utilization of 4 atoms of oxygen and the liberation of 2 moles of carbon dioxide for each mole of acetate. Ajl & Kamen (72) further showed that if doubly labeled acetate is oxidized by *E. coli* suspensions (adapted to oxidize acetate) in the presence of unlabeled pyruvate or of unlabeled dicarboxylic acids, considerable C^{14} accumulates in all initially unlabeled substrates, except α-ketoglutarate. Entirely different results were obtained with *Micrococcus lysodeikticus*, which attacks acetate readily: equilibration of labeled acetate with unlabeled dicarboxylic acids occurred to the same extent with all substrates, including α-ketoglutarate. These results with intact *E. coli* cells are to be interpreted cautiously in view of the results of Novelli & Lipmann (57) and of Stern *et al.* (55, 63) who found that extracts of *E. coli* could synthesize citrate from oxaloacetate and acetate (or pyruvate). On the other hand, as emphasized by Krampitz (73), inasmuch as the orderly conversion of citrate to succinate through the intermediates of the Krebs cycle has not been demonstrated in bacteria, it would appear unwise to make the assumption that this cycle is operative in these organisms.

It is of interest to note in this connection that *E. coli*, known as a non-utilizer of citrate, does grow in Koser's citrate medium and decomposes citrate if certain supplementary compounds are added to the medium [Vaughn *et al.* (74)].

Cutinelli, Ehrensvärd & Reio (75), by growing *Rhodospirillum rubrum* under anaerobic conditions on chemically identical but differently labeled media, could follow quantitatively the assimilation of acetate. The two following groups of compounds were utilized: $C^{13}H_3C^{14}OONa + NaHCO_3$, $CH_3COONa + NaHC^{14}O_3$. The authors found that 45 per cent of the total carbon of the bacterial protein is derived from the methyl group of acetate, 23 per cent from the carboxyl group, and 10 per cent from the bicarbonate pool of the medium.

The chemistry of coenzyme A.—Lipmann *et al.* (76) gave the details for the isolation and the purification of coenzyme A from hog liver. A highly purified preparation contained 11.3 per cent pantothenic acid, 9.1 per cent phosphorus, 20 per adenine, 22.4 per cent ribose, 6 per cent cystine (estimated microbiologically), and probably some glutamic acid. Much of the adenylic acid is probably a contaminant. De Vries *et al.* (77) obtained a very highly purified preparation of coenzyme A (15.4 per cent pantothenic acid, 6.7 per cent phosphorus) from *Streptomyces fradiae*. Glutamic acid was practically absent from this preparation.

Utilizing the synthesis of acetylcholine by the isolated intestine of the rabbit as a test for coenzyme A, Cohen, Cohen-Bazire & Minz (78) had previously observed that washed suspensions of *Clostridium saccharobutyricum* synthesized what they thought to be coenzyme A in the presence of glucose, pantothenate, and glutamate. No other amino acid could replace glutamate. They inferred that glutamate was part of the coenzyme A mole-

cule. However, the test utilized has since been recognized by the authors to be unspecific (79). An explanation for the facts observed remains to be found.

McRorie, Masley & Williams (80) and Brown, Craig & Snell (81) discovered that *Lactobacillus bulgaricus* factor (LBF) contained pantothenic acid in a bound form and the latter authors found that this growth factor is identical or closely related to the product formed when coenzyme A is treated with intestinal phosphatase. De Vries *et al.* (77) found that, as in the case of coenzyme A, LBF contained a sulfur compound resembling cystine. Very recently, Snell *et al.* (82) synthesized a compound which possesses the full activity of LBF and which contains pantothenic acid linked through its carboxyl group with aminoethanethiol, $NH_2—CH_2—CH_2—SH$. Since King & Strong (83) have prepared pantothenic acid phosphates, there is no doubt that more precise knowledge of the chemistry of coenzyme A can be expected in the near future.

Heterotrophic fixation of carbon dioxide.—Attempts by Gerhardt & Wilson (84) to replace the added carbon dioxide required by some strains of *Brucella abortus* by compounds associated with the Krebs cycle or by arginine have failed.

In experiments using C^{13}-labeled bicarbonate, Wessman & Werkman (85) showed that carbon dioxide fixation by lysed cells of *M. lysodeikticus* is prevented by avidin. The addition of adequate amounts of biotin restores the activity. This is presented as conclusive evidence that biotin functions as coenzyme in carbon dioxide fixation, a conclusion already supported by previous work in several laboratories during the past years. However, experiments by Blanchard *et al.* (86) with *Lactobacillus arabinosus* support the view that biotin does not function in carbon dioxide fixation as a prosthetic group or a component of such a group, but rather that it is involved in the synthesis of the enzyme systems of carbon dioxide fixation. This view is supported by various experiments, among which it is interesting to note the need of biotin for the adaptive formation of the "malic" enzyme by *L. arabinosus* and the very slow activation by biotin of the malate dissimilation system of biotin-deficient cells of this organism, as compared to the high activation by nicotinic acid of nicotinic acid-deficient organisms, under otherwise identical conditions.

Herbert (87) obtained highly purified preparations of oxaloacetic decarboxylase ($Q_{CO_2} = 1{,}200{,}000$) from cells of *M. lysodeikticus* by treatment with crystalline lysozyme and fractionation by acetone and ammonium sulfate. Although crystals have been obtained, they are not homogeneous in the ultracentrifuge. The enzyme requires a divalent cation for activity, manganese being the most effective. The purified enzyme is not activated by biotin and all attempts to demonstrate a prosthetic group have failed so far. A mixture of oxaloacetic decarboxylase, malic dehydrogenase, DPN, and manganese, with ferricyanide as hydrogen acceptor, brings about the oxidative decarboxylation of malate to pyruvate and carbon dioxide. The author suggests that carbon dioxide fixation might be due to the reversal of this

reaction sequence. He failed (88), however, to obtain the reductive carboxylation of pyruvate under similar conditions.

Results by McManus (89) preclude malate as an intermediate in this system: in contrast to results with pigeon liver (90), an aqueous extract from disintegrated cells of *M. lysodeikticus* fails to fix $C^{14}O_2$ into malate, even in the presence of triphosphopyridine nucleotide, although good fixation occurs in oxaloacetate.

The possibility of a direct pathway from pyruvate to oxaloacetate in the carbon dioxide fixation reaction is thus not completely ruled out, although experiments by Ochoa *et al.*, using enzyme preparations from pigeon liver (91, 92, 93) and from *L. arabinosus* (94), point to the contrary. Korkes, Del Campillo & Ochoa (94) partially purified the adaptive "malic" enzyme of *L. arabinosus*, which catalyzes the oxidative decarboxylation of malic acid (95). Adaptation leads simultaneously to the production of a decarboxylating activity towards oxaloacetic acid. Both activities seem to be associated with a single protein unit. The over-all reaction

$$\text{L-malate} \rightleftarrows \text{lactate} + CO_2 \qquad \text{(G)}$$

appears to be the result of the sum of two reactions, one due to the "malic" enzyme, catalyzing the reaction

$$\text{L-malate} + DPN_{ox} \overset{(Mn^{++})}{\rightleftarrows} \text{pyruvate} + CO_2 + DPN_{red} \qquad \text{(H)}$$

the other due to lactic dehydrogenase, always present as a contaminant in the preparations, catalyzing the reaction

$$\text{pyruvate} + DPN_{red} \rightleftarrows \text{lactate} + DPN_{ox}. \qquad \text{(I)}$$

The "malic" enzyme of *L. arabinosus* thus appears similar to that of pigeon liver except that it is DPN-dependent instead of TPN-dependent. Experiments by Harary *et al.* (96) further showed that no fixation in oxaloacetate was found when the *L. arabinosus* preparations were allowed to decarboxylate oxaloacetate in the presence of $C^{14}O_2$, irrespective of the presence or absence of ATP, indicating that the decarboxylation of oxaloacetate is not reversible.

Nossal (97) finds that the malic decarboxylase of *L. arabinosus* is activated by yeast extract plus glucose. Yeast extract can be fully replaced by manganese plus potassium ions. An analogous action of potassium ions on a similar enzyme system of *Moraxella lwoffi* had been previously described by Lwoff & Ionesco (98).

Bacterial photosynthesis.—The biochemistry of photosynthesis will not be examined in detail, since it has been reviewed recently by van Niel (99). The relationship of photosynthesis with hydrogenase activity of *Rhodospirillum rubrum* and of *Rhodopseudomonas gelatinosa* was discussed by Kamen (100) during a symposium on chemical transformations in photosynthesis (101). Siegel & Kamen (102) isolated a new strain of *R. gelatinosa* which metabolizes photosynthetically isopropanol, acetone, methylethyl-

ketone, acetoacetate, propionate, and acetate. By isotopic-labeling techniques, isopropanol was shown to be oxidized to acetone by direct dehydrogenation. Simultaneous adaptation experiments conducted by Siegel (103) showed that acetone assimilation occurs through condensation of acetone and carbon dioxide to form acetoacetic acid which is then assimilated. Since the metabolism of acetate is not catalyzed by an adaptive enzyme, it has not been possible to ascertain whether acetoacetate is incorporated as such or is first hydrolyzed to acetate.

Hydrogenase—Billen & Lichstein (184) found that the production of hydrogenase by *E. coli* is conditioned by the presence of certain amino acids in the growth medium. An analysis of the activities of isolated amino acids shows that glutamic acid and methionine are the most effective. This is to be related to the work by Gest & Kamen (185) with *Rhodospirillum rubrum* where this organism produced hydrogen photosynthetically in anaerobiosis only if glutamic and fumaric acids were present.

Joklik (186) purified the hydrogenase from *E. coli* extracts. The enzyme is fairly heat-resistant and is unaffected by dialysis or dilution. Different dyes were tried as hydrogen acceptors: the activity depends neither on the chemical similarity nor on the oxidation-reduction potential, of the dye. Nitrate is not reduced. Full activity is obtained in atmospheres of 15 to 20 per cent hydrogen in nitrogen, where the enzyme is saturated. The enzyme is strongly activated if preincubated in a hydrogen atmosphere, the activation reaching a maximum after 4 hr. of incubation. This is explained as a probable reduction of the enzyme itself: oxygen inactivates the enzyme completely, the activity being fully restored by incubation in hydrogen, or by the addition of various chemical agents. The same worker (187) has observed that compounds known to inhibit sulfhydryl-enzymes also inhibit hydrogenase, the effect being suppressed by thioglycollic acid or cysteine. Hoberman & Rittenberg (188) and Lascelles & Still (189) had previously shown that hydrogenase of intact cells of *P. vulgaris* and *E. coli* was strongly inhibited by hydroxylamine and hydrazine. This inhibition is absent when cell-free extracts are used. The features of the inhibitions observed with cyanide and carbon monoxide give little suport to the theory that hydrogenase is an iron porphyrin enzyme. The actual hydrogen acceptor for hydrogenase, a carrier or perhaps another enzyme system, remains unknown: DPN and cytochrome-*c* are not reduced and flavoprotein is reduced only very slightly.

Nitrogen Metabolism

Assimilation of amino acids.—Najjar & Gale (104) further investigated the passage of lysine across the cell wall of *Streptococcus faecalis* and studied the influence of phosphate ions and of certain amino acids in growing cultures and in washed suspensions on inward and outward migration of this amino acid.

While glutamic acid (105) will enter the cell of *Staphylococcus aureus*

only if glucose is simultaneously fermented, N-phosphoryl-glutamic acid and diethyl glutamic ester enter the cell in the absence of glucose and give rise to free internal glutamic acid.

Deaminases of microorganisms.—Of 27 amino acids and related compounds, Gerhardt, Levine & Wilson (106) found that a strain of *Brucella abortus* oxidizes and deaminates at appreciable rates only L-glutamate, L-asparagine, D-and L-alanine. Practically no ammonia is formed anaerobically.

Wood & Gunsalus (107) previously obtained and partially purified cell-free serine and threonine deaminases from *E. coli*. The resolution of the enzyme shows that it requires adenylic acid and glutathione for activity. The results suggest that both substrates may be activated by a single enzyme.

The function of biotin in aspartic acid, serine, and threonine deaminases has been further studied. Lichstein (108) already emphasized that biotin exists in a coenzyme-bound form in certain natural materials. He separated by paper chromatography of yeast extract a substance which activated the deaminases of serine, threonine, and aspartate (109). This substance liberated biotin upon hydrolysis. Wright, Cresson & Skeggs (110) confirmed the results of Lichstein & co-workers.

Wright *et al.* (111) succeeded in isolating a few milligrams of a crystalline material which is a naturally-occurring complex of biotin, which they have called biocytin, and which behaves as a single substance. Lichstein, Christman & Boyd (112) reported that a concentrate of biocytin is inactive in the aspartic acid deaminase of *Bacterium cadaveris*, while their coenzyme fraction is active. On the contrary, Wright *et al.* (113) find that biocytin is as active as biotin in reactivating the aspartic acid deaminase system previously inactivated. Lichstein (114) finds that the same biotin-coenzyme fraction that is active in the deamination of the three amino acids is also active in the oxaloacetate decarboxylase and succinate decarboxylase systems of *Propionibacterium pentosaceum*.

Paretsky & Werkman (115) found that a strain of an *Achromobacter* species oxidizes glycine with formation of one mole of ammonia per mole of glycine. Hydrogen peroxide is formed during the oxidation; formaldehyde (characterized as the dimedon derivative) is an intermediate of glycine breakdown and undergoes a transformation to formic acid and presumably methylene glycol. Formic acid is completely oxidized while the compound assumed to be methylene glycol is assimilated and transformed to carbohydrate-like material.

The ammonia producing capacity of *Corynebacterium renale* has been investigated by Lovell & Harvey (116) by the use of cell suspensions and cell-free extracts. They found this ability to be linked with the urease activity of the organism which possesses also arginase and uricase.

Sakan & Hayaishi (117) produced evidence that α-hydroxytryptophan is not an intermediate between tryptophan and kynurenine in *Pseudomonas* species, since it is not metabolized by tryptophan-adapted cells or by cells grown in the presence of hydroxytryptophan, while tryptophan-adapted cells oxidize both tryptophan and kynurenine.

Lacombe (118) investigated the breakdown of creatine by *Pseudomonas eisenbergii*. Urea and sarcosine are formed, the latter being further deaminated. Creatinine and glycocyamine are not degraded by this organism. The breakdown of creatinine by *Pseudomonas ovalis* has also been studied. Urea and ammonia are formed; since the organism contains no urease, ammonia is thought to be formed through glycocyamine. Creatine and glycocyamine are also broken down by this organism. The role of metals as activators of these reactions has been investigated. Kopper & Robin (119) found that the degradation of sarcosine by *Pseudomonas aeruginosa* yields glycine and formaldehyde. Cohen, Nisman & Raynaud (120) found that various bacteria break down choline with the production of trimethylamine and ethylene glycol, and ethanolamine with the production of ammonia and ethylene glycol. Certain strains degrade both choline and ethanolamine, while others act only on one of the two substrates. This suggests the existence of two different enzymes. A similar enzyme degrading choline to the same products has since been found by Cromwell (121) in the leaves of *Chenopodium vulvaria* and obtained in the cell-free state.

Stickland reaction.—A peculiar type of coupled deamination between amino acids, known as the Stickland reaction, has been found in *Clostridium sporogenes* (122, 123). A similar reaction has been found by Clifton (124) to occur in *C. botulinum* and by Nisman, Raynaud & Cohen (125, 126) in several other species of anaerobic bacteria.

It is interesting to note that Woiwod & Proom (127) found certain species of anaerobes, namely *Clostridium bifermentans*, *C. sporogenes* and *C. sordellii*, capable of producing δ-aminopentanoic acid in the growth medium, probably by the opening of the proline ring in a Stickland reaction. None of the other species of *Clostridia* tested produced this acid; most of these species had been listed (125, 126) as unable to carry out a Stickland reaction, while the three δ-aminopentanoic acid producing *Clostridia* can carry out such a reaction.

Wagner, Meyer & Dozier (128), in their study of the volatile acids produced by cultures of *C. botulinum*, had characterized acetic, butyric and "valeric" acids as metabolic products. Cohen-Bazire, Cohen & Prévot (129) have shown that it is impossible in such mixtures to distinguish safely between isobutyric, *n*-valeric, isovaleric and *n*-caproic acids by the conventional Duclaux method. By the use of washed suspensions of *C. sporogenes*, *C. valerianicum*, and *C. caproicum*, they have shown that these organisms can carry out Stickland reactions with valine, leucine and isoleucine as hydrogen donors. If glycine is used as hydrogen acceptor, the amount of acetic acid formed renders it impossible to characterize the other acid by the Duclaux method. If however, proline is used instead of glycine, the non-volatile δ-aminopentanoic acid is obtained and isobutyric, isovaleric and optically active valeric acid can be characterized as products of the corresponding amino acids. The data show that the reactions are analogous to that of the classical Stickland reaction between alanine and glycine.

As shown by Saissac, Raynaud & Cohen (130), if these organisms are

grown in the presence of an excess of glucose, no branched acids are found in the cultures, the products being then butyric and acetic acids, resulting from a true glucidic fermentation. Nisman *et al.* (131, 132, 133) have discovered that *C. sporogenes* and other anaerobes can utilize oxygen as an hydrogen acceptor for the oxidation of amino acids, α-keto acids and other substrates. They have characterized the α-keto acids formed aerobically with washed suspensions of anaerobes from the following substrates: alanine, valine, leucine, isoleucine, norleucine, phenylalanine, methionine, serine, threonine and cysteine. The natural isomers only are oxidized. The α-keto acids corresponding to six amino acids have been isolated as the 2,4-dinitrophenylhydrazones. The α-keto acids are themselves aerobically decarboxylated and yield the corresponding volatile acids. No hydrogen peroxide is formed, as shown by the oxygen uptake balance in the presence of catalase or of catalase plus ethanol. The authors propose a scheme of the aerobic degradation of amino acids by strict anaerobes, which involves the presence of two enzymes: an amino acid dehydrogenase of the L-amino acid oxydase type and an α-keto acid dehydrogenase (132).

Following these studies, Nisman & Vinet (134, 135) have found evidence that the enzymes degrading amino acids aerobically and those implicated in the Stickland coupled deamination reaction are identical: if the oxidation of a hydrogen-donor amino acid *d*(alanine, leucine) takes place in the presence of both oxygen and a hydrogen-acceptor amino acid *a*(glycine, proline), the oxygen uptake is 40 to 65 per cent lower as compared with the oxygen uptake without the hydrogen-acceptor amino acid. The excess oxygen consumed in the case of d-O_2 corresponds roughly to the amount of acceptor amino acid reduced in the case of d-a-O_2. This shows a competition for hydrogen between oxygen and the physiological hydrogen acceptor. If sodium arsenite is added to the system d-a-O_2, the oxygen uptake is restored to the value observed in the presence of d-O_2, and the quantity of ammonia formed shows that only the donor is metabolized. This last experiment indicates that arsenite inhibits the reduction of the acceptor amino acid. Further, arsenite has no effect on the reduction of methylene blue by the donor amino acid in the presence of suspensions of *C. sporogenes* whereas it inhibits the reoxidation of leuco-phenosafranine by the acceptor amino acid. Additional evidence lies in the fact that the hydrogen consumption by suspensions of *C. sporogenes* in the presence of the acceptor amino acid is inhibited by arsenite. These experiments indicate that the Stickland reaction results from the action of three enzymes: amino acid dehydrogenase, α-keto acid dehydrogenase, amino acid reductase.

Transaminases.—Bovarnick & Miller (136) investigated the oxygen uptake of typhus rickettsiae in the presence of various substrates. A rapid oxygen uptake occurs only with glutamate or a mixture of α-ketoglutarate and aspartate. Other amino acids and di- and tricarboxylic acids were not oxidized. The data presented suggest that oxidation of glutamate by rickettsiae proceeds through the citric acid cycle with formation of aspartate as a

by-product due to a transaminase, the presence of which was shown by the authors. Housewright & Thorne (137) studied the formation of L-glutamic acid by *Bacillus anthracis* from aspartic and α-ketoglutaric acids. The transaminase of *B. anthracis* has been obtained cell-free by disruption of the cells with sonic vibrations and precipitation with ammonium sulfate. Pyridoxal phosphate activates the enzyme. The authors also studied the composition of the growth medium on transaminase formation by *B. anthracis;* little or no activity is present when the organism is grown in a synthetic medium.

Cohen (138) had previously concluded that the number of monocarboxylic acids which participate in transamination is far smaller than reported by Braunstein & Kritzmann (139). Cammarata & Cohen (140) using more precise techniques, find now that 22 amino acids in addition to alanine, aspartic, and glutamic acids participate in the transamination reaction catalyzed by pig heart, liver, and kidney. Feldman & Gunsalus (141) independently demonstrated a wide variety of transaminases. in *E. coli*, *Pseudomonas fluorescens*, and *Bacillus subtilis* by means of paper chromatography and specific decarboxylase analysis: 14 aliphatic and cyclic amino acids have been found to serve as amino donors in the presence of α-ketoglutarate as acceptor. The glutamic-tyrosine and glutamic-phenylalanine transaminases have been obtained cell-free from *E. coli*, and pyridoxal phosphate has been shown to act as their coenzyme. These results indicate that transamination is of broader importance in the synthesis of amino acids than was previously thought.

Amino acid decarboxylases.—Billen & Lichstein (142) had previously found evidence of the existence of an aspartic acid decarboxylase in *Rhizobium trifolii* and a strain of *E. coli*. Screening of a number of different organisms has shown [David & Lichstein (143)] that this enzyme is not widely distributed among microorganisms and when present, exhibits a relatively low activity, as measured by the microbiological assay of the β-alanine formed with *S. fragilis*, which does not possess the aspartic acid decarboxylase. There is some correlation between the presence of the enzyme and the ability to synthesize pantothenic acid. Gunsalus (144) has published a review dealing with the decarboxylases and transaminases of microorganisms.

Biosynthesis of amino acids.—Nisman, Raynaud & Cohen (145) observed that when washed suspensions of various *Clostridia* are incubated with pyruvate and ammonium salts, not only alanine but also aspartate and glutamate are detected by paper chromatography. The ammonia fixation on pyruvate is thus not a simple amination. It is suggested that transamination reactions occur, similar to those found by Kritzmann (146) in the synthesis of amino acids from pyruvate and ammonia by liver and kidney.

Fincham (147) studied mutant strains of *Neurospora crassa* deficient in aminating ability: the organisms will grow if supplied with any of a number of amino acids. The evidence strongly suggests that the mutants studied are unable to synthesize glutamic acid from α-ketoglutaric acid and ammonia. The fact that any of a number of amino acids will support growth suggests

that transamination mechanisms may link all these amino acids. Only the two transaminase systems so far recorded in other organisms are found in these mutants, but the recent extension by Cammarata & Cohen (140) and by Feldman & Gunsalus (141) of the transamination reaction to a great number of amino acids may throw light on this problem.

Lascelles & Woods (148) found that the synthesis of serine from glycine and formate by cells of *S. faecalis* R deficient in folic acid and pyridoxal is dependent on the presence of both pteroylglutamic acid and pyridoxal. Formylpteroylglutamic acid is more active than folic acid in this respect. Similar experiments with a strain of *Saccharomyces cerevisiae* requiring *p*-aminobenzoic acid (PAB) for growth show that serine synthesis by washed suspensions of this organism is much increased in the presence of PAB, while folic acid (which does not replace PAB for growth) is inactive. Furthermore, growth experiments with *Leuconostoc mesenteroides* show that full growth may be obtained in the absence of serine, provided that pyridoxal, carbon dioxide and PAB are present in the growth medium and that the glycine concentration is increased 40 times. These results suggest that carbon dioxide may serve as precursor of the one-carbon residue, and indicate another mechanism of carbon dioxide fixation involving vitamin B_6 derivatives. Experiments by Hanke & Siddiqi (149) show that when L-lysine or L-tyrosine is treated with the corresponding bacterial decarboxylase in the presence of $C^{14}O_2$ and the reaction is stopped before completion, the remaining amino acid contains the isotope in its carboxyl group. For a discussion of the problem of reversibility of bacterial decarboxylases, the reader is referred to the excellent review by Gunsalus (144).

Shive (150) observed that both methionine and vitamin B_{12} restore growth of sulfanilamide-inhibited *E. coli*. Davis & Mingioli (151) found that with methionineless mutants of *E. coli* vitamin B_{12} can replace methionine only in strains unable to grow with homocysteine. In other words, vitamin B_{12} appears to function in the methylation of homocysteine to methionine. This conclusion has been reached independently by Gots & Koh (152) with *E. coli* and in nutritional studies with chicks (153) and rats (154). A study of the nutritional requirements of 12 mutants of *B. subtilis* has given indications that in this organism, as in *Neurospora*, homoserine is a precursor of both threonine and methionine [Teas (155)].

Umbarget, Magasanik & Mueller (156) have isolated by ultraviolet irradiation two mutants of strain K-12 of *E. coli*, requiring respectively isoleucine and isoleucine plus valine. They obtained convincing evidence that the corresponding α-keto acids are here, as in *Neurospora*, the immediate precursors of these amino acids.

Cells of *S. faecalis* grown with vitamin B_6 contain D-alanine, whereas cells grown with D-alanine do not synthesize vitamin B_6. This indicates a rôle of this vitamin in D-alanine formation (157, 158). Wood & Gunsalus (159) now find that in D-alanine formation, a racemase is involved for which pyridoxal phosphate serves as the coenzyme. A cell-free racemase has been

obtained by sonic vibration and further purified. The purified racemase has been entirely resolved into its apoenzyme and coenzyme fractions. In the presence of pyridoxal phosphate, it converts D- or L-alanine to the racemic mixture. Transamination reactions do not participate in this reaction. The enzyme is widely distributed among bacteria.

Proteases.—Gorini & Fromageot (160) described conditions under which *Micrococcus lysodeikticus*, previously thought not to be proteolytic, develops a proteinase: agar-broth as culture medium, temperature 26°C., pH maintained at about 8 by addition of 0.8 per cent glucose, three days of incubation, strong aeration. Suppression of the proteinase of the culture medium can be obtained by addition of phosphate, citrate, or oxalate, which are either nontoxic or stimulatory for growth. These results are explained by the fact that the proteinase of *M. lysodeikticus* is active and stable only when bound to a certain quantity of calcium (161). Gorini (162) further found that the activity of cell-free proteases from various bacteria is inhibited by calcium reagents such as fluoride, citrate, oxalate, nitrilotriacetate, ethylenediaminotetraacetate, sodium hexametaphosphate. Immediate addition of calcium protects against the inhibition. If however, calcium is added later, the inhibition becomes irreversible.

The partial purification and the properties of the λ-enzyme of *Clostridium welchii* (*C. perfringens*) Type B have been described by Bidwell (163). This enzyme attacks hide powder, gelatin, casein, haemoglobin, and seracin but not native collagen. Tytell & Hewson (164) purified *Clostridium histolyticum* collagenase and separated it from another proteolytic enzyme previously described (165). Virtanen & Kokkola (166) studied the influence of nitrogen nutrition on the excretion of protease by the gelatin-liquefier *Pseudomonas fluorescens*.

Peptide bond synthesis.—Waelsch and associates (167) have found in cell-free extracts of *Proteus vulgaris* enzymes which catalyze the exchange of the amide group of glutamine and asparagine with hydroxylamine resulting in the synthesis of the corresponding hydroxamic acids. The exchange occurs also with ammonia. The view is expressed that this is not the only function of this enzyme system which may play a rôle in peptide synthesis, and that amides, once formed, may take part in synthetic reactions by transfer of the glutamyl or aspartyl radicals. The same group of workers (168) found that cell-free preparations of the enzyme catalyzed the synthesis of glutamohydroxamic acid from glutamic acid as well as glutamine, but ATP and magnesium were then needed as additional components of the system. The extracts also split added glutamo- and aspartohydroxamic acids and showed glutaminase and asparaginase activities.

Nitrate reduction.—Egami & Sato (169) obtained a cell-free preparation of nitrate reductase by ultrasonic destruction of cells of *E. coli* and studied the properties and the mode of action of this enzyme. They came to the conclusion that cytochrome-*b* of *E. coli* type is identical with nitrate reductase (170, 171). These results lead Egami & Murakami (172) to the consider-

ation that the nitrate reductase-nitrate system plays the analogous rôle to that of the Warburg-Keilin system with nitrate in place of oxygen. The authors make the statement that bacteria without cytochromes can not reduce nitrate at all. This is probably erroneous, since Woods (173) and others have stated that *C. perfringens* and many other obligate anaerobes do reduce nitrate.

Nucleic acid metabolism.—Wang, Sable & Lampen (174) found that extracts of cells of a particular strain of *E. coli* deaminate cytidine to uridine and ammonia by means of an hydrolytic enzyme. Cytosine desoxyriboside is deaminated three to four times as rapidly as cytidine. Cytidylic acid is deaminated only if intestinal phosphatase is added to the bacterial extracts. The enzyme is not inactivated by dialysis or freezing. The Michaelis constants were determined for cytidine and cytosine desoxyriboside. Other purines, pyrimidines, and derivatives were not deaminated.

Paege & Schlenk (175) found that acetone-treated cells of *E. coli* contain a pyrimidine riboside phosphorylase which catalyzes the following reaction:

$$\text{pyrimidine riboside} + \text{phosphate} \rightleftarrows \text{pyrimidine base} + \text{ribose-1-phosphate.} \qquad \text{(J)}$$

The action of this enzyme appears restricted to uridine. With intact cells, uracil is accumulated and the carbohydrate is metabolized rapidly.

Wright *et al.* (176) found that *Lactobacillus arabinosus* 09 requires orotic acid (uracil-4-carboxylic acid) for growth. This requirement can be met partially by ureidosuccinic or by 5-(carboxy-methylidine)-hydantoin. This last compound is chemically related to ureidofumaric acid. This is taken as microbiological evidence that ureidosuccinic and perhaps ureidofumaric acids are precursors of orotic acid. As the orotic acid requirement of the organism can not be met by uracil, cytosine or by their nucleosides or nucleotides, the authors consider the hypothesis that the synthesis of nucleic acid by this organism does not involve the participation of uracil or derivatives, but that orotic acid is directly incorporated into nucleic acid and further decarboxylated. Data obtained from isotopic studies already indicated that orotic acid but not uracil or uridine is the precursor of nucleic acid pyrimidine in the rat (177, 178).

Woolley & Pringle (179) found that when *E. coli* is grown in the presence of amounts of 4-aminopteroylglutamic acid sufficient to inhibit the multiplication, 4-amino-5-carboxamidoimidazole (a probable precursor of hypoxanthine) accumulates as in the case of sulfonamide bacteriostasis (180, 181). This favors the view expressed by Woods (182) that the primary action of sulfanilamide inhibition is the inhibition of pteroylglutamic acid synthesis from PAB, and that the synthesis of purines depends in turn on folic acid.

Pigment synthesis.—Using glycine with labeled carbon and nitrogen, Hubbard & Rimington (183) showed that all three nitrogens of the prodigiosin synthesized by *Serratia marcescens* are derived from glycine. The α-carbon of glycine is also utilized in this synthesis, but not the carboxyl carbon atom. With acetate as the labeled element, they were able to show that both carbons of acetate are utilized in this biosynthesis.

Fermentations

Fatty acid synthesis and butanol-acetone fermentation.—Pyruvate is generally accepted as an important intermediate in most bacterial fermentations. However, the participation of pyruvate in butyric and butanol-acetone fermentations was still in question until recently. The evidence has been reviewed by Cohen (190). According to Cohen & Cohen-Bazire (191, 192), the different results obtained by various authors could be explained by the fact that strains with different properties were utilized. Thus, washed suspensions obtained from certain strains of butyric acid bacteria, called "normal," produce acetate and butyrate from pyruvate, whereas suspensions from other strains, called "deficient," give rise to acetate only from pyruvate. In addition, small amounts of lactate are produced with both groups of strains.

Cohen-Bazire *et al.* (193) found that arsenite suppresses butyric acid synthesis from pyruvate by "normal" strains and does not affect acetic acid formation. Thus, it causes a separation of the condensation of acetate from its formation. Simon (194) had previously found that dried or washed cells of *Clostridium acetobutylicum* produced only acetic acid as a dissimilation product of pyruvate. Rosenfeld & Simon (195) have defined experimental conditions where the fermentation of pyruvate leads to the production of high levels of butyl compounds and acetone. In view of their results, the bacterial preparations previously utilized by Simon (194) have to be considered as incomplete enzyme systems. They find now that in the presence of a very dense bacterial suspension, and of both potassium and magnesium, the yields of acetone from the dissimilation of pyruvate are as high as those obtained from glucose. They also made the interesting observation that in the presence of fluoride, the formation of hydrogen from pyruvate is decreased and the formation of butyl compounds increased.

Along the same lines, results obtained in the reviewer's laboratory (196) have shown that in the presence of ferrous ions, and to a lesser extent of manganous or magnesium ions, "deficient" suspensions become able to produce butyrate from pyruvate. Fluoride, which is without effect on the fermentation of pyruvate by "normal" suspensions, induces also butyrate formation from pyruvate by the "deficient" cells. These results agree with the results of Rosenfeld & Simon (195). The latter authors (197) also studied the fermentation of phosphoenolpyruvate by washed suspensions of *C. acetobutylicum;* the same products are formed as are obtained from glucose and pyruvate. However, the yield of butyl compounds is 2.6 times higher than from pyruvate. The authors discussed the possible role of phosphoenolpyruvate in the butanol-acetone fermentation. Cohen & Cohen-Bazire (198) found that while lactate is not attacked either by "normal" or "deficient" suspensions of butyric acid bacteria, "normal" suspensions synthesize butyrate if incubated with both lactate and acetate. These results are similar to those obtained by Bhat & Barker (199) with growing cultures of *Clostridium lacto-acetophilum.*

There is a great uncertainty regarding the actual structure of the C_4-compounds involved in the synthesis of butyrate by anaerobic bacteria. In this respect, the outstanding work of Stadtman & Barker on the metabolic activities of *C. kluyveri* extracts has been analyzed in Volume 4 of the *Annual Review of Microbiology* (73), but the present reviewer feels that it should be discussed again in relation to more recent work.

Stadtman & Barker (200, 201), working with extracts of *C. kluyveri* and Cohen & Cohen-Bazire (202, 203) utilizing suspensions of butyric acid bacteria, found that acetoacetate is split into two C_2-residues. The latter workers found acetate formed from acetoacetate, while the former observed a phosphoroclastic splitting of acetoacetate to acetate and acetyl phosphate. It should be noted that the suspensions utilized by Cohen *et al.* possess strong acetyl phosphatase activity. Nisman (204), utilizing extracts of the same bacteria obtained by ultrasonic disruption, finds that pyruvate yields considerable amounts of acetyl phosphate, both aerobically and anaerobically.

The reverse reaction, that is the synthesis of acetoacetate from acetate and acetyl phosphate, has been postulated by Lipmann (205, 206) as a step in the synthesis of butyrate from pyruvate. Stadtman & Barker (201), in an attempt to obtain this reaction find only small but nonetheless significant amounts of a β-keto acid which they have not characterized and which could be acetoacetic acid. β-keto acid accumulates in greater quantity in presence of 0.002 M cyanide.

Incubation of extracts of *C. kluyveri* with acetate, acetyl phosphate, and hydrogen leads to a synthesis of butyrate (67), while acetoacetate incubated with hydrogen under the same conditions gives rise, almost mole for mole, to β-hydroxybutyrate, which is not further metabolized, and to traces of butyrate (201). In view of these results, Stadtman & Barker (201) come to the conclusion that acetoacetate is not a precursor of butyrate. This assumption is supported by further experiments conducted by the same workers with C^{14}-labeled compounds. On the other hand, Cohen & Cohen-Bazire (202, 203) showed that suspensions of butyric acid bacteria may utilize lactate or pyruvate as hydrogen donors for the reduction of acetoacetate to butyrate. The "deficient" suspensions give rise only to acetate from pyruvate or acetoacetate; simultaneous incubation with both pyruvate and acetoacetate leads to the formation of butyrate. In view of Stadtman and Barker's experiments, the author (207) studied the reduction of acetoacetate by molecular hydrogen. Since Cohen *et al.* (191, 203) have found β-hydroxybutyrate to be fermented *in vacuo* to acetate and butyrate, its behavior in a hydrogen atmosphere was also studied. Contrary to what occurs *in vacuo*, β-hydroxybutyrate is not metabolized in an atmosphere of hydrogen. This is consistent with Stadtman and Barker's observations. In the case of acetoacetate, 25 to 30 per cent is reduced to β-hydroxybutyrate; this also agrees with the results of these authors. Most of the remainder is split to acetate, while a small amount only is reduced to butyrate. The

possibility cannot be excluded that this small amount of butyrate is derived from a previous splitting of acetoacetate followed by a recondensation of two C_2-residues to another C_4 intermediate. The utilization of sodium arsenite has allowed Cohen *et al.* (207) to discount the possibility of a preliminary splitting of acetoacetate to acetate. Arsenite inhibits this splitting. It also inhibits almost completely at a convenient concentration, the fermentation of β-hydroxybutyrate (203), which is fermented *in vacuo* by way of acetoacetate, the suspensions possessing an active β-hydroxybutyric dehydrogenase (208). In the presence of acetoacetate, hydrogen, and of a convenient concentration of arsenite: (*a*) the ratio of hydrogen consumed to acetoacetate metabolized is more than doubled; (*b*) traces only of β-hydroxybutyrate are produced; (*c*) practically no splitting of acetoacetate occurs and butyrate is the main if not the only product of the reaction. The results of Cohen & Cohen-Bazire are visualized by the following scheme:

$$\begin{array}{c} 2\ CH_3COOH \leftarrow CH_3COCH_2COOH \rightarrow X(?) \rightarrow CH_3CH_2CH_2COOH \\ \qquad\qquad \uparrow\downarrow \\ \qquad\qquad CH_3CHOH\ CH_2COOH. \end{array} \qquad (K)$$

In the absence of arsenite, three reactions compete for the utilization of acetoacetate: splitting to acetate, reduction to β-hydroxybutyrate, reduction to butyrate. The data obtained demonstrate that in the presence of arsenite, the two first reactions are inhibited, two moles of hydrogen being utilized per mole of butyrate produced. The over-all reaction in the presence of arsenite is:

$$CH_3COCH_2COOH + 4H \rightarrow CH_3CH_2CH_2COOH + H_2O. \qquad (L)$$

This reduction may involve an intermediate in the same state of oxidation as β-hydroxybutyrate, this compound itself being definitely excluded as an intermediate in butyric acid synthesis by the organisms studied.

In vacuo, the reaction

$$CH_3COCH_2COOH + 2\ H \rightleftarrows CH_3CHOHCH_2COOH \qquad (M)$$

proceeds towards the left, while in an atmosphere of hydrogen, the equilibrium is shifted to the right and β-hydroxybutyrate is not fermented. These results by no means allow a definite statement as to whether acetoacetate is actually an intermediate in butyric acid synthesis by bacteria. Nevertheless, they show that it can be reduced to butyrate. It is the reviewer's opinion that the problem should be reinvestigated.

Stadtman & Barker (209) continued their studies on fatty acid synthesis by cell-free extracts of *C. kluyveri*. They showed that these preparations catalyze a rapid and probably direct reversible transfer of the phosphoryl group of acetyl phosphate to adenosinemonophosphate and adenosinediphosphate. The same preparations catalyze also the transfer of the phosphoryl group of acetyl phosphate to fatty acids possessing three to six carbon atoms. These transphosphorylations are not mediated by the adenylic acid system. The mechanism of the reduction of fatty acids to the

corresponding alcohols has been investigated for the first time: the authors found that the acyl phosphates of acids from propionic to caproic are reduced to the corresponding alcohols. Butyryl phosphate is apparently not involved in the synthesis and oxidation of caproate. A very active transacetylating enzyme was found that transfers the acetyl group of acetyl phosphate to inorganic phosphate or arsenate.

Seeley & VanDemark (210) obtained and partially purified a cell-free preparation of the acetoacetic decarboxylase of *C. madisonii*. They partially resolved the purified enzyme by digestion with papain, trypsin, and alkaline phosphatase. The resolved enzyme is stimulated greatly by the addition of a boiled extract of the original acetone powder preparation.

Other fermentations.—Delwiche (211) and Johns (212) had previously brought convincing evidence that propionate formation by propionic acid bacteria and by *Veillonella gazogenes* proceeds through decarboxylation of succinate. Delwiche (213) reports that biotin-deficient propionic acid bacteria have a greatly lowered succinic acid decarboxylase activity and that activity can be restored upon addition of biotin to the washed biotin-deficient cells.

Leaver (214) has included C^{14}-formaldehyde in the growth medium of *Propionibacterium arabinosum* and observed that radioactive carbon was incorporated in the resulting propionic acid. The three carbons of propionate contained radioactivity, in contrast to what occurs with carbon dioxide which is fixed only in the carboxyl group of propionate (215). Furthermore, the higher specific activity of the carboxyl group indicates that formaldehyde was incorporated in the carboxyl position of propionate by some mechanism in addition to carbon dioxide fixation. It is of interest that Wood & Werkman (216) previously identified formaldehyde in the fermentation medium of propionic acid bacteria. Formaldehyde thus appears as a possible essential intermediate in the propionic acid fermentation.

It has been previously proposed by Krampitz & Everett (217) that α-acetolactic acid, $CH_3COH(COCH_3)COOH$, was an intermediate of acetylmethylcarbinol formation from pyruvate. Juni (218) has now demonstrated that cell-free extracts of various bacteria are able to form acetylmethylcarbinol and carbon dioxide from pyruvate and to decarboxylate α-acetolactic acid. A crude extract of *A. aerogenes* has been resolved into two fractions, one acting on pyruvate to produce α-acetolactic acid and carbon dioxide, the other without action on pyruvate, but decarboxylating α-acetolactic acid to acetylmethylcarbinol. The first component depends on cocarboxylase. The second is specific for α-acetolactic acid and does not decarboxylate oxaloacetate or acetoacetate.

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METABOLISM OF FUNGI

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This review covers papers which have appeared since the manuscript of the book "Chemical Activities of Fungi" [Foster (50)] was completed, up to March 1951. Failure to cover certain papers of significance is due to unavailability of the particular journals.

A person seeking a trend in the field of metabolism of fungi will be especially impressed with the number of papers dealing with mutations and biochemical synthesis, with degradation of high molecular weight polymers of many kinds, and with organic acid formation. A new text, "Physiology of Fungi" by Hawker (69), unavailable to this writer, but reviewed by Gray (67), was published in 1950. The broad significance of mold metabolism was stressed by Sparrow (137) in an address entitled "The Expanding Horizons of Mycology."

Biochemical Genetics

The flow of comprehensive papers on biochemical genetics of Neurospora continues unabated [Horowitz (75); Emerson (41); Bonner (16); Mitchell (102)] to the point that one reviewer [Glass (64)], while recognizing features of value, wonders if such frequent comprehensive articles are essential. Since the present review is perforce literature coverage, the writer will assume similar criticisms do not apply here.

Mutations and techniques.—Attention has been recently given to the influence of the experimental methods on the number and types of mutants obtained in biochemical genetics. The question has been raised, "Do the methods determine the abundance and the nature of mutants obtained?" Lein *et al.* (88) revealed that of 500 presumptive *Neurospora* conidiospore mutants obtained by ultraviolet radiation, 250 were lethals of some type since they germinated but failed to develop further on a complex complete medium. Mitchell (102) considers this group to include mutants requiring substances that will not penetrate the cell wall, such as macromolecules like proteins, nucleic acids, vitamin conjugates, and many phosphorylated compounds. Two hundred and fifteen mutants grew slowly for unknown reasons, whereas only 35, less than 10 per cent of the total, represented identifiable deficiency types.

Furnishing a complete medium automatically precludes obtaining certain kinds of deficiency mutants, those which are sensitive to inhibitory substances in the complex medium that do not affect the wild type. In this category so far are histidine, tryptophan, thiamine, and methionine mutants of *Neurospora* [Lein *et al.* (88)], guanine mutants of *Ophiostoma* (*Ceratostomella*) *multiannulatum* [Fries (57)], and a riboflavin mutant of *Aspergillus niger* [Foster and Norman (54)]. Indole was shown to be the inhibitor of the

tryptophanless mutant; adenine and hypoxanthine of the guanine mutant; and in the remaining cases, unknown substances were responsible. Numerous other instances are known in which amino acid-less mutants are sensitive to inhibition by other amino acids. On the other hand, Fries (56) observed that deficiency mutant conidia of *Ophiostoma* are more viable than wild type in deficient media, yielding fallaciously high mutagenic rates.

Horowitz (75) has analyzed available data on temperature mutants to show that the methodology of biochemical genetics is not particularly selective for biochemical deficiency type mutants exclusively. His analysis supports the one gene-one function-one enzyme theory of gene action as contrasted to the idea that a single gene performs more than one function.

Whereas *Neurospora* has been the favored fungus for biochemical genetics, it obviously has no advantage over the use of deficiency mutants of other organisms from the standpoint of biochemistry. Consequently, there is an increasing tendency toward use of other fungi (see below) as well as bacteria [Davis (33)], in biochemical mutation work.

New mutagenic chemicals of proved use with fungi now include substances such as mustard gas, caffeine [Fries (59)], organic peroxides [Dickey *et al.* (38)], and possibly indole [Cushing, Schwartz & Bennett (31)]. The indirect mutagenic action of radiation via H_2O_2 has been established for *Neurospora* [Wagner *et al.* (157)]. The mutagenic action of *tert.*-butyl hydroperoxide and a peroxide isolated from diisopropyl ether on reversion of adenineless *Neurospora* spores was approximately ten times greater than H_2O_2 [Dickey *et al.* (38)], and the authors suggest that the mutagenic action of radiations occurs via the intermediary intracellular formation of organic peroxide, the latter being the immediate mutagenic force.

Combination treatments to secure increased mutation rates ought to be valuable for expediting the production of rarer deficiency mutants. Sequential infrared and ultraviolet irradiation is known to increase mutation rate, and pretreatment of *Neurospora* microconidia with a relatively nonmutagenic concentration of nitrogen mustard alters the stability of the genes in such a manner as to increase materially the action of ultraviolet light in producing both morphological and biochemical deficiency mutants [Swanson, McElroy & Miller (146)].

Tatum, Barratt, Fries & Bonner (147), in a comprehensive study of induced mutations, concluded that *bis-* and *tris*-nitrogen mustards, ultraviolet, and x-ray radiations are about equally mutagenic for *Neurospora* and that mutation rates are the same regardless of the isolation technique employed. In general, the kinds of mutants obtained via the different mutagens are the same, and the conclusion was made that the stability of the particular genes is more important than the type of mutagen, and the mutation pattern will by and large always be the same for a given organism. Thus, regardless of mutagen, the most frequent deficiency mutant(s) of *Escherichia coli* is proline; of *Bacillus subtilis*, cystine, methionine, histidine, and arginine; of *Saccharomyces cereviseae*, leucine, lysine, and methionine;

of *Ustilago maydis*, adenine, hypoxanthine, thiamine, and nicotinic acid [Perkins, (113)]; of *Penicillium notatum*, arginine, lysine and methionine; of *Ophiostoma multiannulatum*, arginine, lysine, methionine, purine, and pyrimidines [Fries (58)]. However, killing curves of *Chaetomium globosum* indicate differential mechanisms for x-rays and ultraviolet, the data indicating that five quantum hits of x-rays or two of ultraviolet at 2,804 Å are necessary for a kill [Ford & Kirwan (49)]. Also, x-rays did not produce any K type morphological mutants, whereas ultraviolet did do so characteristically in large numbers. With respect to production of penicillin by *Penicillium chrysogenum* Q176, ultraviolet irradiation produced greater numbers of variants than did nitrogen mustard, and with the former, the shift was toward mutant strains of lower penicillin potency, whereas with mustard, the shift was toward mutant strains of higher penicillin potency [Reese, Sanderson, Woodward & Eisenberg (126)]. Spontaneous changes in *P. chrysogenum* Q176 were found only rarely.

One gene-one enzyme theory.—While this theory is regarded as acceptable in most quarters, a shift in viewpoint appears fairly well established. In deficiency mutants with a genetic block, it has been assumed that the interruption of a biosynthetic sequence was due to the loss of the specific enzyme at the block in the mutant. This usually implied that the mutated gene did not allow synthesis of that particular enzyme. While there may be truth in this statement, it is clear at present that this is not the sole interpretation of gene action. Wagner & Guirard (156) and Wagner (155) worked with a pantothenicless *Neurospora* mutant that differed from wild type by inability to synthesize the vitamin from the pantoyl lactone and β-alanine precursors, and therefore presumably lacked the enzyme for this reaction. Acetone-dried mutant mycelium did, however, effect the reaction, permitting the conclusion that the enzyme was indeed present but in an inactive state in the intact cell. Here, then, the gene action is not involved in the production of the specific enzyme for the reaction, but instead exerts an indirect influence on the enzyme synthesized independently. A reasonable assumption is that the mutation results in formation of a substance which inhibits the particular enzyme. Several analogous types of inhibition sensitivity in *Neurospora* are known, the best example perhaps being Zalokor's sulfonamide-requiring mutant. One may visualize that the gene mutation may indeed affect directly only a single enzyme, which may result in greater or lesser synthesis of some substance; this enzyme or its gene need not, however, bear any direct relation to the pantothenic enzyme or its gene.

Such models support the one gene-one enzyme theory, with the qualification that the gene and its enzyme may create a phenotype in which the lack of an essential action bears only a chance relation to the actual mutated gene. Altered rate of synthesis of a particular substance as a direct result of a gene mutation obviously could cause inhibition of, or alterations in, several enzymes simultaneously; several phenotypical manifestations may therefore appear simultaneously as a result of a single gene mutation. In

the past, this condition has been used in postulating the existence of multifunctional genes. However, on the basis of shunt metabolism and interrelated metabolic pathways [Foster (50), Emerson (41)] not only is the phenomenon adequately explained, but becomes the expected behavior, because the presence or absence of a single substance may disturb directly or indirectly numerous processes in the cell. The observation that biochemical genetics has a unique value in studying biosynthesis because " . . . it is possible by its means to isolate and study vital metabolic processes in the living organism without disturbing in an observable way any of the other processes of the cell" [Horowitz (75)] is subject to dispute.

Cushing, Schwartz & Bennett (31) have an interesting illustration of the value of *Neurospora* mutants for the study of the genetic basis of adaptive phenomena. After several transfers through indole-containing media, a single gene mutant was isolated which grew faster than wild type in 10^{-3} *M* indole. The indole resistance was not due to a superior ability to metabolize indole. It was suggested that indole itself induced the observed mutation.

Mechanisms of biosynthesis.—Blastocladia has been found to require methionine as a sulfur source [Cantino (24)] and the related *Allomyces arbuscula* requires either cystine or methionine [Ingraham (79)]. Such findings invalidate the statement recently made (50) that specific amino acid requirements among fungi are not known. The ability of fungi to utilize sulfate sulfur for growth and synthesis of organic sulfur compounds is almost universal, and in the above organisms the biosynthetic sequence from inorganic sulfate evidently has been genetically disrupted in nature.

Extensive use of biochemical mutants has been made for the study of biosynthesis of the sulfur-containing amino acids, but almost exclusively from the standpoint of the origin of the carbon chains [see Horowitz (75) for summary]. Consideration is now being given to the pathway of conversion of sulfate to organic sulfur. Essentially this is reduction, and has an importance also for complete reduction of sulfate to sulfide in biological systems, particularly by sulfate-reducing bacteria. It has generally been assumed that sulfate reduction follows the inorganic sequence: sulfate, sulfite, sulfoxylic acid, thiosulfate, sulfide, and evidence for the stepwise genetic control of the first two stages has been obtained with *Neurospora* mutants [Horowitz (75)]. Evidence also is accumulating that sulfate reduction occurs via an organic pathway; indeed, transformations of inorganic substances in nature, both reductive and oxidative, via organic pathways may well be a general method. In the *Neurospora* mutants mentioned above, cysteic acid appeared to be metabolically equivalent to sulfite, and cysteine sulfinic acid to thiosulfate, indicative of successively reduced levels of organic sulfur.

Hockenhull (71) concluded from nutritional studies of 13 cystineless mutants of *Aspergillus nidulans* that H_2S was not an intermediate in conversion of sulfate to organic sulfur and postulated the following sequence for the assimilation of sulfate by *A. nidulans:* Sulfate, sulfite, sulfoxylate (hypothetical), thiosulfate, $HSO_3 \cdot S \cdot CH_2 \cdot CH(NH_2)COOH$ (cysteine S-

sulfonate or serine thiosulfate), $HSCH_2CH(NH_2)COOH$ (cysteine). The fission of cysteine S-sulfonate yielding cysteine was considered analogous to the fission of alkyl thiosulfates by *Penicillium brevicaule* and *Schizophyllum commune* yielding methyl and ethyl mercaptans [Challenger & Briscoe in (71); see also Challenger & Lowther (29)]. Heterocaryon studies indicate that three genes are implicated in each of the changes sulfate to sulfite and sulfite to thiosulfate. Starkey (142) gives a comprehensive review of the transformations of organic sulfur compounds by microorganisms in the soil, in which sulfate was reported to be the principal end product of cystine metabolism by certain fungi. This oxidation presumably is the reverse of the reductive processes described above.

Nitrogen metabolism.—The mechanism of biological nitrate and nitrite reduction and assimilation is receiving close attention; the evidence, though preliminary, indicates the intermediary participation of organic compounds of nitrogen in a manner analogous to the metabolism of inorganic sulfur compounds described above. In nitrifying bacteria, ammonia assimilation and oxidation may occur via the glutamic acid-glutamine system [Quastel & Scholefield (119)]. Nitrate and nitrite reduction in *Neurospora* involves organic nitrogen compounds [De la Haba (35)].

Amino acids and vitamins.—Only papers published subsequent to the reviews of Horowitz (75) and of Mitchell (102) will be covered here.

The sulfonamide-requiring *Neurospora* mutant has been further studied by Zalokar (165); the sulfonamide gene controls a deleterious reaction. Five methods are now known for growth of the sulfonamide mutants. Strehler (145) postulated that *p*-aminobenzoic acid functions in the methylation of homocysteine-thiolactone to methionine in *Neurospora*.

The relationship of proline to the arginine cycle in *Neurospora* has been strengthened by the discovery that ornithine and proline each have a common precursor, α-amino-δ-hydroxyvaleric acid: proline⇄α-amino-δ-hydroxyvaleric acid⇄ornithine⇄citrulline⇄arginine [Srb, Fincham & Bonner (138)]. The precursor does not arise exclusively from proline, but also via another unknown mechanism, and furthermore, it was not active in similar *Penicillium chrysogenum* mutants. Arginine synthesis in *Ophiostoma multiannulatum* has been found to be the same as in other systems, i.e. via ornithine and citrulline [Bergström & Sjöbeck (10)].

Lysine biosynthesis in *Ophiostoma* mutants proceeds as follows: unknown precursor⇄α-keto-adipic acid⇄α-aminoadipic acid⇄lysine [Bergström & Rottenberg (9)], whereas another sequence appears to occur in *Neurospora:* unknown precursor⇄α-aminoadipic acid⇄ε-hydroxynorleucine⇄lysine [Good, Heilbronner & Mitchell (65)].

Two organic acids accumulate in culture filtrates of an isoleucineless-valineless *Neurospora* mutant, and not in wild type filtrates [Adelberg & Tatum (2)]. These have been identified as the corresponding α, β-dihydroxy analogues of the two amino acids, and are considered as precursors in the synthesis of the latter. The valine precursor also supports growth of a valineless *E. coli*.

From work with indole-N^{15} as a precursor of nicotinic acid in *Neurospora*, Bonner & Wasserman (17) found that only one-fourth of the vitamin arose via the labeled precursor. Lack of equilibration between exogenous and endogenous indole is indicated. A complex relation between hexachlorocyclohexane (gammexane) and inositol has been found with an inositolless mutant [Fuller, Barratt & Tatum (60)], a study of which indicated that gammexane inhibits utilization of exogenous inositol only.

Adaptation of the PAB-less *Neurospora* mutant No. 1633 for the assay of this vitamin in natural extracts has been accomplished [Agarwalia & Peterson (3)]. The organism does not respond to pteroylglutamic acid, and the basal medium is so complex that nonspecific stimulation by organic extracts is excluded.

Lipids.—Lein & Lein (86, 87) have studied the lipid metabolism of *Neurospora* mutants. One mutant requires oleic, linoleic, or linolenic acid, but cannot use long-chain saturated fatty acids. An acetate-requiring mutant can utilize in place of acetate a number of naturally occurring fatty acids; presumably the organism secures the needed acetate by breakdown of the fatty acids, and this offers a technique for ascertaining if acetate is an intermediate in the breakdown of larger molecules by *Neurospora*.

Sterol synthesis in *Neurospora* has been studied with C^{13}-labeled acetate [Ottke and coworkers (109, 110)]. Acetate proved to be the principal precursor of fat and sterol in an acetateless mutant. Over 90 per cent of the carbon atoms of cellular ergosterol and fatty acids originated from acetate, the methyl carbon and the carboxyl carbon of which were utilized to the same extent in the synthesis of the lipids. The methyl carbon of isovaleric acid also participated slightly in sterol synthesis.

Carotenoids.—β-carotene was thought to be the only carotenoid-synthesized by *Phycomyces blakesleeanus;* however, Schopfer & Grob (132) discovered that depending on the medium used, five carotenoids may be produced. They found the carotene localized in the large yellow fat globules within the cells. Acetate added to a lactate medium led to high production of β-carotene, therefore acetate was postulated as the precursor. Garton, Goodwin & Lijinsky (61) found only β-carotene in *P. blakesleeanus,* and its synthesis by the mold grown in the dark was only one half that in light; total fat synthesis and dry weight were meanwhile unaffected by light. Substitution of glycine for asparagine in glucose-salts medium led to marked stimulation of synthesis of β-carotene.

The leaves of the crabapple tree infected with pycnidial lesions of the common rust fungus *Gymnosporangium juniperi-virginianae* have the high content of 83.2 mg. carotene per 100 gm. dry weight. Of this, the exceptionally high level of 34.5 per cent was γ-carotene [Smits & Mitchell (136). No γ-carotene was found in uninfected leaves.

Decomposition of Polymers

Starch.—The superiority of mold-produced amylase over malt as a saccharifying agent in industrial alcohol fermentation of grains, and the

tremendous economic implications has led to a rapid exploitation of this subject. Selected strains of *Aspergillus niger* are cultivated in the submerged state and whole culture added to a corn mash in proportion of 1 to 11 per cent by volume. The ethanol yields after secondary yeast fermentation were 90 per cent of theory, as compared to 80 to 85 per cent for mold bran hydrolysis and 70 to 74 per cent by the conventional barley malt hydrolysis [Teixeira, Andreasen & Kolachov (148)]. A flow sheet and other aspects of a pilot plant process for production of ethanol from grain saccharified with mold amylase is available [Le Mense *et al.* (84)].

Maltase and amylase are formed independently of one another in various mold cultures [Gates & Kneen (63)], and dextrinase and amylase are formed adaptively and specifically [Hultin & Nordström (77)]. Using a new viscometric method, the latter authors concluded that dextrinase attacks all the 1,6 linkages in the substrate molecule with equal ease and does not degrade the molecule from its ends.

Weaver & Cordon (159) discovered that a considerably greater recovery of the amylolytic enzymes in mold cultures than that in the filtrate alone could be accomplished by releasing the ample amounts of enzymes retained in the mold cells. Physical comminution of the hyphae, or autolysis induced by killing agents such as merthiolate or ammonium bifluoride released maltase bound in both living and dead fungus cells.

Crystalline α-amylase and crystalline limit dextrinase have been isolated from *Aspergillus oryzae* filtrates by $(NH_4)_2SO_4$ precipitation procedures [Underkofler & Roy (153)]. The former represents the first isolation from a fungal source.

Cellulose.—Cellulose attack by a lignin decomposing fungus was favored by the presence of pentosans and lignin, suggesting that the decomposition of these substances individually in the laboratory may not reveal the true rates possible in nature [Heuser *et al.* (70)]. Generally, lignin fungi and cellulose fungi are highly specific for their respective substrates. Decomposition of cellulose in nature is not limited to cell wall remains of dead plants; cellulose fibers of the cotton boll and of raw cotton in general are subject to attack by cellulolytic fungi, and a recent survey indicates such attack (cavitomic cotton) is rapidly expanding geographically in cotton-producing areas of the south [Elting (39)]. This particular report contains excellent photomicrographs of cotton fibers deteriorating under the influence of fungi.

Amorphous precipitated cellulose particles are more rapidly degraded than cellulose in micellar structure by cell-free enzymes from *Tricholoma fumosum* and *T. nudum.* Based on turbidity of precipitated cellulose suspensions, a photometric method for measuring cellulolytic enzyme activity was developed [Norkrans (108)]. Respirometric studies have been applied to cellulose decompostion by a homogeneous suspension of *Myrothecium verrucaria* cells [Darby & Goddard (32)]. This organism does not ferment glucose, and 65 to 90 per cent of the carbohydrate consumed is oxidatively assimilated aerobically. Reese & Downing (125), in a comprehensive approach to cellulose decomposition by fungi, list a number of pretreatment

factors influencing subsequent fungus decomposition of the cellulose. A useful practical classification key was constructed for the identification and differentiation of the fungi most commonly associated with cellulose and with wool decomposition. These authors conclude that all species of *Aspergillus* can hydrolyze the linkage between anhydroglucose units in straight-chain molecules derived from cellulose; the difference between cellulose decomposers and nondecomposers is in the ability to carry out the earlier hydrolysis of the cellulose by the cellulolytic enzyme.

Wool.—White, Mandels & Siu (161) present a comprehensive review of destruction of wool by molds. Most organisms which can attack native wool are related to pathogenic dermatophytes. There appears to be no relation between keratinolytic activity, wool decomposition, and cellulose decomposing ability of various fungi. Growth of fungi is always better on autoclaved wool, due to partial breakdown of wool proteins; after autoclaving, numerous ordinary fungi not considered as wool fungi, will grow on the chemically degraded wool.

A considerable advance in the biochemistry of wool degradation by the fungus *Microsporium gypseum* has been contributed by Stahl and co-workers (139, 140, 141). Inorganic sulfate is an end product of wool decomposition. Evidence was obtained for sulfate formation from wool cystine via the following sequence ($R = -CH_2CHNH_2COOH$): $2RSH \rightleftarrows R\text{-}S\text{-}S\text{-}R \rightleftarrows RSH + RSOH$ (sulfenic acid); $RSOH \rightarrow RSO_2H$ (sulfinic acid) $\rightarrow RH + SO_3H_2$; $SO_3H_2 \rightarrow H_2SO_4$. *M. gypseum*, *Scopulariopsis brevicaulis*, and *A. niger* each could degrade methionine at the sufur linkage, yielding methyl mercaptan. Soluble nitrogen is released during wool decomposition by *M. gypseum;* 50 per cent of this fraction is ammonia. The fungus evidently conducts a differential hydrolysis of wool because the undigested wool residues decrease in sulfur and in cystine content. The primary reactions in wool deterioration by this organism are therefore proteolytic. Deamination of a polypeptide occurs after primary hydrolysis of the keratin molecule. Simple autoclaving of wool increases its amino-nitrogen content. *M. gypseum* was found to be the most active of several species in digesting keratin materials like cow's horn, fingernails, wool, and human hair [Page (111)]. This paper contains some excellent photomicrographs of the digestion of cow's horn and of the attack of human hair by the mold.

Amino acid oxidases.—D- and L-amino acid oxidases of *Neurospora* have been shown to differ in substrate specificity and in many other respects [Barker & Krebs (7)]. The D-amino acid oxidase of *P. chrysogenum* Q176 has been described [Emerson, Puziss & Knight (40)].

Neutral fats and fatty acids.—A stable lipase for triacetin was extracted from anhydrous defatted *Fusarium lini* mycelium and a 15 fold purification accomplished [Fiore & Nord (46)]. Electrophoretically, the preparation contained several components. Free sulfhydryl groups were found not to be essential for lipase activity. The existence of different types of fungus lipases is suggested by the fact that the extracellular and intracellular lipases

of *A. niger* and *Penicillium roquefortii* have different pH optima [Fodor & Chari (47)]. Shipe (134) gives cogent evidence for different lipases produced by the two fungi just mentioned, based upon relative substrate specificity of the enzymes. Marked differences were observed in the relative rates of hydrolysis of five neutral glycerides. Also, the activities of the enzymes were affected differently by free butyric, caprylic and oleic acids.

Thaler and co-workers (149, 150) provide more evidence that methyl ketone formation from saturated fatty acids during rancidity occurs via β-oxidation of the fat acid. If the β-carbon is blocked by methylation the acid is not attacked, whereas α-methyl substituted acids are attacked and the corresponding series of methyl ketones produced. The expected methyl ketones were produced from each type compound in the postulated fatty acid degradation sequence, i.e., from the α,β-unsaturated acid, the β-hydroxy acid, and the β-keto acid. Decarboxylation of the latter yields the methyl ketone.

Lignins.—*Trametes pini* was found to contain 23.7 per cent sulfuric acid lignin [Schubert & Nord (133)]. Pelczar, Gottlieb & Day (112) have studied lignin utilization by various white rot fungi. Their results with *Polyporus versicolor* represents the first instance of lignin utilization as the sole source of carbon by a fungus in synthetic medium; the fungus grew almost as well as it did on glucose.

Pectin.—Fungal (*Byssochlamys fulva*) polygalacturonase has been obtained free from pectin esterase [Holden (74)] and a pectinase type enzyme has for the first time been obtained free from pectin methoxylase. The molecular size of pectin is reduced without the production of galacturonic acid, i.e., only nonglycosidic bonds are ruptured.

Organic Acids

The last few years have seen a more rigorous and direct biochemical approach to mechanisms of formation of organic acids by fungi than formerly. Walker's (158) general review on this subject should be consulted.

Oxalic acid.—Most noteworthy is the work of Lynen & Lynen (91), who proposed that oxalate formation from acetate (produced from sugar) by *A. niger* occurs via a modified tricarboxylic acid cycle. The intermediate steps postulated involved condensation of acetate with oxaloacetate to form citrate, followed by oxidation to oxalosuccinate. The oxalosuccinate is then cleaved to oxalate and succinic acid. The latter is oxidized to oxaloacetate via fumaric and malic acids; condensation of the C_4 acid with another acetate begins a second cycle. Evidence for a split of oxalosuccinate to yield oxalate and succinate is lacking. The entire theory is based on the finding that the percentage of theoretical yield of oxalate was the same whether citrate, fumarate, succinate, or acetate was used as the sole substrate. Despite the attractiveness of this hypothesis, it cannot be considered as proved; yield data at best can only eliminate possible mechanisms, but by themselves seldom can conclusively prove any one mechanism.

Lewis & Weinhouse (89), using acetate-1-C^{14}, obtained labeled citrate and oxalate. In line with Lynen & Lynen's hypothesis, they assumed citrate to be a precusor of oxalosuccinate and oxalate, though no evidence for this mechanism was advanced. When labeled carbon dioxide was present with unlabeled glucose or acetete, labeled oxalate was produced. Since C_6 and/or C_4 carboxylic acids are known precursors of oxalate, and themselves can be produced from either acetate or glucose, it is plausible to assume that carbon dioxide entered via metabolic exchange with carboxyl groups of the carboxylic acids [Foster & Carson (51a)]. Carson & Foster have shown in unpublished experiments that the C_4 dicarboxylic acids isolated during oxalate production from glucose in the presence of C^{14} labeled carbon dioxide were labeled in the carboxyl groups. The oxalate was also labeled. The oxalate formed by *A. niger* mycelium from labeled fumaric acid as the sole substrate had a specific radioactivity approximately equal to that of the starting fumarate. Since the radioactivity of the fumarate was distributed between α-carbon and carboxyl-carbon in the ratio 4.2:1, the indications are that the oxalate arose from a central split of the C_4 chain, presumably after oxidation to oxaloacetate.

Recurring proposals for the origin of oxalate from acetate via glycolate and glyoxylate [Smith (135)] contain no more conclusive or critical evidence than existed previously.

Fumaric acid.—The formation of fumarate from C^{14}-labeled ethanol by washed cell suspensions of *Rhizopus nigricans* has been described [Foster *et al.* (52)]. The α-carbons of fumarate had the same specific radioactivity as the 2-carbon of ethanol when ethanol-2-C^{14} was used as the substrate. With ethanol-1-C^{14} as a substrate, the α-carbons of fumarate were devoid of radioactivity, and the carboxyls were labeled. These results were interpreted as supporting the Thunberg-Wieland condensation: 2 acetate $\xrightarrow{-2H}$ succinate $\xrightarrow{-2H}$ fumarate. Labeled carbon dioxide appears in the carboxyls of the C_4 dicarboxylic acids of *R. nigricans* with unlabeled ethanol as the substrate. It was concluded that C_4 dicarboxylic acids originating by one or more carbon dioxide-independent mechanisms can acquire radioactivity in the carboxyls from labeled carbon dioxide without a net synthesis of the acid from carbon dioxide. Unpublished data (Jefferson, Anthony, Phares, Carson & Foster) show that C^{14}-formate in a glucose fermentation of *R. nigricans* becomes located in each carbon of the fumarate, succinate, and malate molecules formed by this organism, as well as in the ethanol. Little can be said of the mechanism at this time except that it may have consequences for the Thunberg-Wieland mechanism proposed above.

Bernhauer & co-workers (100, 123, 124) have developed a submerged process for production of fumarate by *Rhizopus*. Yields between 50 and 60 per cent of sugar consumed are obtainable. Selenium salts and fluoride suppress fumarate formation and favor ethanol accumulation whereas *p*-benzoquinone (0.1 per cent) increased the fumarate yield in one experiment from 52.2 up to 67 per cent, at the expense of ethanol and lactate.

Citric acid.—Martin, Wilson & Burris (96), using $C^{14}O_2$ in the atmosphere over *A. niger* producing citric acid from glucose, found the radioactivity exclusively in the carboxyl groups of the isolated citrate and distributed equally between the primary and secondary carboxyls. They computed that 5.5 per cent of the citrate-carbon originated from carbon dioxide. Still unanswered is the question of the relative importance of new synthesis of citrate via a net carbon dioxide uptake reaction such as pyruvate plus carbon dioxide or α-ketoglutarate plus carbon dioxide, as contrasted to carbon dioxide exchange with acids preformed by a carbon dioxide-independent mechanism. Foster & Carson (51) have proposed a possible mechanism for citrate synthesis involving condensation of 2 molecules of acetate-2-C^{14} to yield a C_4 dicarboxylic acid; this is oxidized to OAA which condenses with a third molecule of acetate yielding citrate. The origin of oxaloacetate via a carbon dioxide-independent reaction was suggested on the basis of a tracer experiment in the relative rates of incorporation of acetate and carbon dioxide carbon into citrate. Computations indicated that only 1.3 per cent of the total citrate carbon originated from carbon dioxide carbon. If carbon dioxide fixation via oxaloacetate or malate were entirely responsible for net oxaloacetate and citrate synthesis, the figure should be 16.7 per cent. An important theoretical objection to making quantitative conclusions in work of this kind with living cells is uncertainty of reaction rates, concentration gradients, and equilibria between reactions; generally some assumptions are necessary. In this case, lack of equilibration between extracellular and intracellular carbon dioxide would lead to apparently low carbon dioxide fixation values. On the other hand, there is an equally good possibility that carbon dioxide to some extent entered the citrate molecule by exchange with preformed oxaloacetate or other acid(s). In experiments of this kind it is difficult to differentiate between a dicarboxylic and a tricarboxylic acid cycle.

Strain specificity may well account for differences in results obtained in work of this type. Martin, Wilson & Burris (96) found no labeled carbon dioxide produced from labeled citrate in the presence of glucose; in a similar experiment, Foster & Carson (unpublished data) found significant labeled carbon dioxide evolution inside of one hour.

The importance of acetate condensation in citrate synthesis is supported by increased yields of citrate when acetate is added to cultures containing sugar [Halliwell (68), Bomstein & Johnson (18)].

Lactic acid.—Bernhauer, Rauch & Miksch (14) describe factors conducive to 70 to 87 per cent yields of *d*-lactic acid from glucose by *Rhizopus delemar* in submerged culture. Iodoacetate (2 mg. per cent) increased the lactic acid yields at the expense of the ethanol normally produced in the cultures. *p*-Benzoquinone behaved similarly and selenic acid conversely. The yields reported in this paper were based on the calcium content of the filtrate; $CaCO_3$ was used as a neutralizing agent during fermentation. The lactate values may be somewhat high, because succinic, fumaric, and malic acids generally accompany lactic acid in these fungi.

Gastel & Gibbs (62) fermented glucose-3,4-C^{14} by a lactic acid *Rhizopus* and found the radioactivity distributed in the lactate as follows: carboxyl-carbon 97 per cent; α-carbon 2.2 per cent; β-carbon 0.8 per cent. It is evident that the lactate arose mainly by glycolysis; nevertheless, the definite radioactivity in the α- and β-carbons indicates some other process must have occurred. Carson *et al.* (26) have given evidence for an oxidative pathway of lactate formation in *Rhizopus*, in addition to the usual glycolysis. Pyruvate is decarboxylated to a C_2 moiety; two of these condense to succinic acid; this is oxidized to oxaloacetate and possibly malate which are reductively decarboxylated to lactate. Oxygen is essential for this process. Unpublished data suggest a third mechanism of lactic acid formation by this mold. When glucose was fermented in the presence of C^{14}-formate, the lactate was found to be labeled in all carbons, but preponderately in the β-carbon.

To the growing list of aquatic Phycomycetes known to produce lactate may be added *Allomyces arbuscula* [Ingraham (79)]. Cantino (23) has found that an elevated carbon dioxide tension in a *Blastocladia pringsheimii* fermentation increased the proportion of succinic acid produced, at the expense of lactic acid. Presumably carboxylation of pyruvic acid occurs here.

Other acids.—Striking results of Arnstein & Bentley (5), confirmed by Denison (36), utilizing $C^{14}O_2$ and $HC^{14}OOH$ as tracers make it evident that these two substances are not precursors of kojic acid during formation of the latter from glucose by fungi. Formation of unlabeled kojate under these conditions raises the possibility of direct conversion of the glucose carbon chain to the kojate carbon chain without previous rupture.

Homogentisic acid is produced from tyrosine by several fungi in yields up to 21 per cent and is said to be a normal intermediate in tyrosine oxidation by these organisms [Utkin (154)]. *Merasmius foetidus* oxidizes gallic acid by an extracellular catecholase, forming an unidentified yellow product [Lindeberg & Korjus (90)]. Gallic acid could not be used as the sole source of carbon, but when added to glucose cultures increased the economic coefficient by 50 per cent. The riboflavin-producing *Ashbya gossypii* displays a typical alcoholic fermentation anaerobically, and an essentially complete oxidation of sugar aerobically [Mickelson (99)].

Food lipid and protein production.—Considerable attention is being devoted to the use of fungi to convert cheap carbohydrates and mineral nitrogen into fat and protein for food and technological purposes. Species of *Fusarium* evidently are superior agents of lipid synthesis, and biochemical aspects of lipid synthesis are reported [Nord, Fiore, Kreitman & Weiss (107); Maselli & Nord (97), Deschampes (37)]. Technological and pilot plant aspects of lipid and protein synthesis by fungi have been extensively studied by Bernhauer *et al.* (12). Strains of *Mucor circinelloides* and *Fusarium bulbigenum-tracheiphilum* were outstanding for fat production. Efficiency of the mold growth decreased with increased sugar concentration; nevertheless, fat content of the mycelium was proportional to the sugar concentration. Up to 5 gm. raw fat was obtained per liter of 4 per cent sugar medium.

Other Chemical Activities of Fungi

Biosynthesis.—Hockenhull, Ramachandran & Walker (72) on the grounds of comparative biochemistry propose that biosynthesis of penicillin proceeds via condensation of L-cysteine, D-β-hydroxyvaline, and a carboxylic acid similar to β-hydroxydimethylpyruvic acid or dimethylpyruvic acid. Synthesis of the latter acid by fungi is known. Production of hydrocyanic acid by an unidentified basidiomycete is described [Robbins, Rolnick & Kavanaugh (129)] and β-nitropropionic acid was isolated from an *Aspergillus flavus* filtrate [Bush, Tooster & Brockman (22)]. The only other naturally occurring nitro compound known is chloramphenicol. Strauss (144) reports an oxidative and a fermentative decarboxylation of pyruvate in *Neurospora*. An acetate mutant apparently can produce acetate via the fermentative mechanism even though the oxidative mechanism is genetically blocked. Various fungi can oxidize $MnSO_4$ to MnO_2, but the autotrophic status of these fungi is undetermined [Bromfield & Skerman (20)]. A polysaccharide isolated from culture filtrates of *Fusarium solani* produces wilt and stem end rot symptoms on potato seedlings, similar to the infection by the pathogen itself [Thomas (151)].

Chemicals produced by fungi.—Citrinin has been synthesized [Cartwright, Robertson & Whalley (27)] and the structure of patulin revised [Engel, Brzeski & Plattner (43); Woodward & Singh (163)].

Illustrative of the variety of organic chemicals synthesized by fungi (polychemism) are homoprotocatechuic acid and 2,4,5-trihydroxyphenylglyoxylic acid from *Polyporus tumulous* filtrates [Ralph & Robertson (121)]; two new polysaccharides produced by *Penicillium lacteum*, one consisting of 88 per cent D-galactose, the other of 64 per cent glucose [Freeman & Macpherson (55)]; and chrysophanic acid by *P. islandicum* [Howard & Raistrick (76)]. The latter substance is 4,5-dihydroxy-2-methylanthraquinone, and bears close chemical relationship to eleven other known products of fungi. The usnic, didymic, and rhodocladonic acids obtained from the lichen *Cladonia cristatella* are now known to be produced by the isolated fungal member of the symbiosis [Castle & Kubsch (28)].

Raistrick (120) has coordinated a great amount of information on complex chemicals produced by fungi by showing relationships in chemical structures. The majority of compounds discussed fall into approximately a dozen groups of basic chemical types.

Ingelman (78) has reviewed the status and transformations of high molecular weight inorganic polymers of phosphate (metaphosphates) which are found in yeast and fungi. Doubtless important functions will be established for this new type of metabolite.

Inhibition of fungi.—The term "funginert," distinct from fungicidal or fungistatic, has been proposed [Ezekiel (45)] to describe manufactured materials, " . . . not supporting fungus growth because the material, part or component in question does not furnish the necessary nutrients for growth." The powerful antifungal antibiotic actadione incorporated into

isolation media suppressed the growth of fungi from natural sources without affecting bacteria [Phillips & Hanel (117)].

A review by McCallan (92) discusses the nature of copper and sulfur fungicide action on plant pathogenic fungi. Exudates produced by fungus spores, containing hydroxy- and amino acids, react with "insoluble" copper fungicides to form soluble copper complexes which are the directly toxic agents. Fungicides containing elementary sulfur also are believed to exert an indirect action. Thus, sulfur vapor is converted to the toxic H_2S by phytochemical reduction in the spores or the host plant tissue. Application of sulfur as a fungicide to plant leaves infected with rust results in acceleration of metabolism of the sulfur above that of uninfected plants [Yarwood & Jacobson (164)]. Selective absorption of sulfur was demonstrated by radioautographs utilizing H_2S^{35} vapors.

Of the synthetic organic fungicides, compounds of the dithiocarbamate class receive particular emphasis in the literature [Miller & Elson (101); Klöpping (80)]. Baldwin (6) concludes that of all the organic sulfur fungicides, the dithiocarbamates, possessing the essential grouping —C(=S)S— are the most active. Manten, Klöpping & van der Kerk (94) describe the chemical and physiological properties of one member of another series (thiuram disulfides) showing marked fungicidal activity. Metabolic studies indicate that specific assimilatory processes rather than dissimilatory are inhibited by this fungicide. Klöpping (80) found that fungicides whose antifungal spectra indicated a high degree of specificity did not inhibit respiration in fungistatically active concentrations, whereas those of low specificity inhibited respiration and growth in the same concentration range.

Certain naphthoquinones inhibit spore germination of *Monilinia fructicola* to a degree parallel to inhibition of yeast carboxylase by the quinones [Foote, Little & Sproston (48)]. It would be premature to accept the authors' interpretation, based on the foregoing, that carboxylase might be the site of inhibition of the fungi by the naphthoquinones; application of the results of enzymatic experiments to intact cells bridges a wide gap. Certain antihistamines (diphenylpyridine and pyribenzamine) are antifungal to dermatophytes and systemic fungi [Carson & Campbell (25)] and the inhibition is reversed by histamine [Landis & Krop (82)].

Ingenious experiments of Brian, Wright, Stubbs & May (19) demonstrated that the chlorine containing antibiotic griseofulvin is taken up by cuts and also through the roots of several plant species, and is distributed systemically. With this technique, 75 per cent protection was obtained against *Botrytis cinerea* infection of lettuce. Work of this type poses the interesting practical question of the edibility, toxicity, etc., of crop food plants protected by systemic antibiotics. No evidence was found for synthesis of griseofulvin by artificial inoculation of a griseofulvin-producing fungus into nonsterile soil, and Björkman (15) could obtain no soil control of the pine root rotting fungus *Polyporus annosus* by artificially inoculated antagonists. Although suggestions of control appear occasionally, the state-

ment made earlier [Foster (50)] viz., "the antibiotic substance as a natural force has yet to be demonstrated," apparently is still valid. This line of reasoning was applied particularly to the balance of microorganisms in nature.

The wood rotting fungus *Schizophyllum commune* attacks only dead American elm trees; living trees contain an inhibitor preventing fungus attack [McDonough, Bell & Arnold (93)]. The inhibitor is rapidly inactivated after the tree dies.

Trace elements.—Trace elements in fungus growth, metabolism, and industrial processes have been examined in numerous papers [Erkama and co-workers (44); Tomlinson, Campbell & Trussell (152); Bernhauer, Rauch & Gross (13); Jarvis & Johnson (79a); Perlman (114)].

Physiological aspects of zinc uptake by *Neurospora crassa* have been studied in detail [Andersson-Kottö & Hevesy (4)], and enzymatic consequences of zinc deficiency have been described. Zinc deficient *Rhizopus nigricans* is virtually devoid of pyruvic decarboxylase although this particular enzyme is not a zinc protein, nor does it require Zn^{++} for activation [Foster & Denison (53)]. Zinc deficient *Neurospora* shows considerable disturbance in content of various enzymes; some, such as tryptophanase and alcohol dehydrogenase, are markedly reduced or eliminated, and others such as diphosphopyridine nucleotidase are markedly increased, in comparison with zinc sufficient mycelium [Nason (104); Nason, Colowick & Kaplan (105)]. It is clear that synthesis of various enzymes is interrelated and that zinc influences the synthesis of some enzymes. It is therefore not surprising that the pattern of the zinc effect should be different in different organisms. Deficiencies of other trace elements induce results similar to zinc deficiency, with respect to citric acid formation in *A. niger* [Chesters & Robinson (30)]. Nickel and cobalt toxicity for *A. niger* is reversed by magnesium [Abelson & Aldona (1)].

Nutritional, physiological, cultural, or other aspects.—General reviews are available covering physiological and biochemical factors influencing dormancy and germination of mold spores [Gottlieb (66)], the sexual hormones in *Achlya* [Raper (122)], the mycological aspects of *Ashbya gossypii* in relation to riboflavin production [Pridham & Raper (118)], the growth of fungi in synthetic solutions [Steinberg (143)], and mycological aspects of penicillin [Perlman (115)]. An excellent comprehensive survey of the present status of mold processes in industrial microbiology is available [Lee (85)], and the nutrition of 310 Basidiomycetes has been surveyed by Robbins (127).

The problem of pleomorphism in dermatophytic fungi is considered by Robbins & McVeigh (128) to be due to mutation, and not segregation of a heterocaryon into mycelial and spore genetic types as recently proposed [Wilhelm (162)]. The results and interpretations reported in these two papers have yet to be reconciled. In the case of *Histoplasma capsulatum*, the yeast-like phase is favored over the mycelial phase by the presence of

cystine or methionine [Salvin (131)], that of *Blastomyces dermatiditus* by 37° incubation [Nickerson & Edwards (106), Salvin (130)], and that of several yeast-like fungi by the presence of 15 to 20 per cent carbon dioxide in the atmosphere [Bullen (21)].

Biotin can be replaced entirely by oleic acid plus aspartic acid for *Ophiostoma pini* [Mathiesen (98)] but not for a cholineless mutant of *Neurospora* [Hodson (73)]. The concept of "association" in mycology has been effectively developed by Westerdijk (160) for the specific sequences and combinations of fungi occurring in decaying biological substrates, and the intrinsic and extrinsic factors involved are detailed [Mossel & Westerdijk (103)].

The nitrogenous fractions of *A. niger* mycelium and autolysates contain heterocyclic compounds similar in amounts and types to soil humus [Küster (81)]. A specific phenol oxidase is present in white wood rotting fungi, but is absent in the brown rots [Law (83)]. The geographic distribution of *A. niger* strains has been studied, with the conclusion that freshly isolated strains produce more citric and oxalic acids than stock cultures [Peters & Rippel-Baldes (116)]. Markert (95) found that the fertility of the cross between two strains of Glomerella depends on diffusible chemical substances secreted by those strains. The paper by Emerson (42) demonstrates what the power of statistics can do for limited and selected data.

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NUTRITION OF BACTERIA AND FUNGI[1]

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Introduction

An attempt to condense and evaluate a voluminous literature in the brief space allotted to this review necessitates the deletion of many papers, noteworthy as they may be. It is inevitable, too, that selection of topics must reflect the perhaps prejudicial interest and interpretation of the reviewers. Although one cannot over-estimate the immense contributions that have been made through the use of microorganisms for the determination of amino acids, vitamins, and growth factors, this subject has been reviewed extensively (1 to 6) and will be omitted here.

At first glance it would appear that microbial and animal nutrition have advanced via diverse pathways; however, a closer examination reveals that the two have always been intertwined (7). The advancement of knowledge in both fields has resulted primarily from a gradual transition from the use of simple or natural media or diets to ones of increasingly precise chemical definition, as one vitamin or growth factor after another is identified. Since the gaps in the obvious growth factor requirements for many animal species have in general filled in more quickly, increasing effort has been directed toward elucidating the apparently more fastidious needs of microorganisms. This is the field of endeavor with which we will be concerned, and one can only be amazed at the rapidity of the advances here. Yesterday's unidentified growth factor, in some natural material, becomes identified and frequently synthesized today, and almost simultaneously is shown to play some important role in the physiology or metabolism in higher forms of life. From such discoveries, the concept has developed that the metabolic reactions of all living organisms are basically similar.

Thiamine, Niacin, and Riboflavin

The well-established role of these vitamins in the nutrition of many microorganisms and their general use in synthetic media is attested to by the fact that very few recent papers have been concerned with the nutritional role of these substances. Of interest, however, is a report (8) that *Hemophilus piscium* requires a thermolabile factor, not coenzyme 1, which is required by certain other members of this genus. Griffin has shown (9) that the growth activity of this thermolabile factor can be replaced by either diphosphothiamine or adenosinetriphosphate (ATP) at levels of 1 and 30 μg. per ml. of medium respectively. Thiamine itself at levels from 0.1 to 10

[1] The survey of literature pertaining to this review was concluded in March, 1951.

mg. was inactive, and in fact at high levels was shown to inhibit the activity of cocarboxylase in a manner suggestive of competitive inhibition (10).

Through the use of *Neurospora crassa* mutants, the metabolic transformation of tryptophan to niacin has been greatly clarified. This serves to emphasize the important role of microorganisms in analyzing metabolic interrelationships evident also in higher forms.

Georg (11) has shown that the fungus *Trichophyton equinum* requires either niacin or niacin amide for growth. Interestingly, this fungus responds to L-tryptophan in the absence of niacin, although the vitamin is about 2,500 times as active on a weight basis as the amino acid.

Riboflavin may be replaced by its diethyl analogue, and at limiting levels and for short growth periods the latter serves equally well as a growth factor for *Lactobacillus casei*. If the incubation period is extended to 120 hr., riboflavin is superior to diethyl riboflavin in allowing acid production [Lambooy (12)].

Pantothenic Acid, Coenzyme A, and the Lactobacillus Bulgaricus Factor (L.B.F.)

The discovery by Lipmann *et al.* (13) of the relation of coenzyme A to pantothenic acid and its role in numerous acetylation reactions has stimulated great interest in pantothenic acid nutrition in both bacteria and animals. It is evident from the analyses of animal tissues and cells of microorganisms, that essentially all of the pantothenic acid present exists in bound forms such as coenzyme A [Novelli *et al.* (14)]. King and co-workers (15) have reported another form of pantothenic acid existing as a conjugate (PAC) in the nondialyzable fractions of tissues. Along with pantothenic acid, the conjugate contains glutamic acid and adenine. Like coenzyme A, PAC is inactive for lactic acid bacteria, although pantothenic acid can be liberated from it by the action of alkaline phosphatase and an unidentified liver enzyme. Pantothenic acid conjugate, however, possesses much greater growth activity than pantothenate itself for *Acetobacter suboxydans*, *Aerobacter aerogenes*, and *Escherichia coli;* it also has greater activity than the free vitamin for overcoming the inhibitory effect of certain analogues of the vitamin for *A. suboxydans* [King & Cheldelin (16)].

Since most vitamins are thought to function in the cell in the form of phosphate derivatives, King & Strong (17) have prepared various phosphates of pantothenic acid in a series of ingenious syntheses and have tested their biological activities. It was found that like the bound forms of pantothenic acid, i.e., coenzyme A and PAC, the synthetic phosphates of the vitamin were inactive for *Lactobacillus arabinosus* until after treatment with intestinal phosphatase to liberate pantothenic acid. Considering the extreme sensitivity of free pantothenic acid to alkaline treatment, it is most interesting that the pantothenic acid phosphates retain their vitamin activity after treatment with normal NaOH in a boiling water bath for 1 hr. This is pertinent to the earlier observation from the same laboratory that an alkaline stable form of pantothenic acid exists in natural materials.

Hoff-Jørgensen *et al.* (18) have reported a factor found in highest concentration in yeast extract, although present in other natural materials, which is required not only for several strains of *L. bulgaricus* but for several other strains of lactic acid bacteria that have complex growth factor requirements (19). When the concentration of pantothenic acid was increased to a rather high level in comparison to the amounts needed by other lactic acid bacteria, maximum growth was obtained without addition of the L.B.F. (20, 21). Concentrates of the L.B.F., however, were more potent than pantothenic acid, on a comparable solids basis. It was concluded by Brown *et al.* (21) that L.B.F. contains bound pantothenic acid which is "probably either identical with or closely related to the product formed when coenzyme A is treated with intestinal phosphatase" (14).

It has been shown recently by Snell *et al.* (22) that L.B.F. may be N-(pantothenyl)-β-aminoethanethiol or the corresponding disulfide. This compound was synthesized and appears to be indistinguishable from the L.B. factor. Pantetheine and pantethine were suggested as names for the sulfhydryl and disulfide forms of the synthetic compound.

Long & Williams (23) have noted that the L.B. factor may exist in several forms. By paper chromatographic separation and the bioautograph technique, seven forms of the L.B. factor have been shown to exist. Although L.B.F. contains pantothenic acid, there appears to be some question whether all forms of L.B.F. are derived from coenzyme A.

It is obvious that much more work remains to be done before the complex interrelations of the bound forms of pantothenic acid are clarified.

Biotin

Biotin has been associated with the metabolic fixation of carbon dioxide and the synthesis of aspartic acid [Lardy, Potter & Burris (24)]. The specificity of biotin is attested by the fact that homobiotin (antimetabolite of biotin) inhibits aspartic acid synthesis. Although Lichstein & Umbreit (25) have shown that biotin restores the deaminase activity of aged bacterial cells, yeast extract contains a fraction that is considerably more potent than biotin itself. This coenzyme factor, free from biotin, contains a bound form of the vitamin that becomes available to *Saccharomyces cerevisiae* after acid hydrolysis (26). Liver, as well as yeast, contains this coenzyme of aspartic acid, serine, and threonine deaminases. The substance liberated from this coenzyme fraction replaces biotin for the growth of *S. cerevisiae* and is not dextro-biotinic acid (27).

The lack of uniform response obtained with *L. arabinosus* and *L. casei* when yeast extract is assayed prior to acid hydrolysis [Wright & Skeggs (28)] appears to be resolved. Though acid hydrolysis of yeast results in an increased biotin value with *L. arabinosus*, the value found with *L. casei* remains unchanged. This combined form of biotin which yields additional biotin activity for *L. arabinosus* subsequent to acid hydrolysis has been isolated in crystalline form by Wright *et al.* (29) from yeast. The prodigious task involved is indicated by the fact that only a few milligrams of crystal-

line material were obtained from several tons of yeast. This material (m.p. 203 to 210°C. with decomposition), named biocytin, yields about 40 per cent of biotin as measured microbiologically after acid hydrolysis.

Biocytin seems to be different from the biotin-containing coenzyme factor in liver and yeast because of its essential inactivity in the aspartic acid deaminase of *Bacillus cadaveris*. Furthermore, its biological activity with respect to the growth of certain strains of yeast is markedly different from that of the coenzyme itself [Lichstein, Christman & Boyd (30)].

The inability of the cholineless mutant of *N. crassa* to utilize oleic acid, aspartic acid, or Tween 80 in place of or along with biotin, marks a difference in the metabolism of biotin between lactic acid bacteria and Neurospora (31). It has been suggested by Williams & Fieger (32) that oleic acid is able to eliminate the requirement for biotin because of its surface activity. This might in part explain the difference in response to oleic acid between Neurospora and the lactic acid bacteria.

Desthiobiotin and *o*-heterobiotin only partially replace the biotin requirement for the growth of *Allescheria boydii*. This organism differs from lactic acid bacteria in that oleic and aspartic acids, etc., are not utilized for growth [Villela & Cury (33)].

Oxybiotin, at pH 7.0 or above, is about 30 per cent as effective as biotin in causing growth and aspartic acid synthesis in *L. arabinosus*. Growth and aspartic acid synthesis are inhibited as the pH is lowered and this effect is more evident with oxybiotin than with biotin (34). Oxybiotin, if given in high concentration, has been shown to be as effective as biotin in stimulating the bacterial deamination of aspartic acid (35).

Broquist & Snell (36) have supplied further evidence of marked differences in the metabolic pathways concerned with biotin in different microorganisms in that biotin plays no role in the synthesis of aspartate by *Lactobacillus fermenti* and *Clostridium butyricum*. For the lactic acid organisms, *L. arabinosus*, *L. casei*, and *Streptococcus faecalis*, aspartic acid and unsaturated fatty acids are needed for growth when biotin is completely absent, but for *L. fermenti* and *C. butyricum*, unsaturated fatty acids alone allow growth under similar conditions.

Alterations in the structure of biotin have in general resulted in inactive compounds or metabolic antagonists. To obtain further information regarding the effects of changes of the biotin molecule on its activity, guanido analogues of *d*-biotin and *dl*-oxybiotin have been synthesized by Hofmann & Axelrod (37). These compounds are practically without activity for *S. cerevisiae*, *L. arabinosus*, and *L. casei*, which re-emphasizes the biological specificity of the cyclic urea portion of biotin and oxybiotin.

Pyridoxine

All three members of the vitamin B_6 group, pyridoxine, pyridoxal, and pyridoxamine, have been found in natural materials. These compounds are differentiated by the fact that the yeast *Saccharomyces carlsbergensis* re-

sponds to all three compounds, while for *Streptococcus faecalis*, pyridoxal and pyridoxamine are about 1,000 times as active as pyridoxine. *L. casei*, on the other hand, is able to utilize only pyridoxal [Rabinowitz & Snell (38)].

It was shown by McNutt & Snell (39, 40) that *Lactobacillus helveticus*, *L. acidophilus*, and *L. delbrueckii* required pyridoxamine phosphate or pyridoxal phosphate for growth, despite the fact that normally adequate amounts of pyridoxal and pyridoxamine were present in the medium. Pyridoxamine phosphate proved to be 6 to 8 times more active than pyridoxal phosphate.

Attempts by these workers (40) to extend their observations made it evident that factors, even in addition to those mentioned above, were needed for adequate growth of these bacteria. Considerable experimentation revealed that the addition of desoxyribosides, reducing agents, or vitamin B_{12} was essential for growth and with the addition of any one of these materials, the organisms again responded to the phosphates of pyridoxal and pyridoxamine in the same manner as observed previously.

L. delbrueckii, which in contrast to the other two organisms requires thymidine for growth, cannot use vitamin B_{12} or reducing agents in place of the desoxyriboside.

It was also found (40) that *L. helveticus* and *L. delbrueckii* could utilize D-alanine but not L-alanine for growth in place of phosphorylated vitamin B_6 compounds. This extends the observations made with many organisms that D-alanine replaces vitamin B_6 when the media contain all the other compounds which are synthesized through the mediation of vitamin B_6. This is interpreted to mean that D-alanine is required by these organisms, but can be synthesized in the presence of B_6.

The need for phosphorylated forms of B_6 appears to result from a loss of phosphorylating mechanisms by the organism; it also implies that considerable variation is possible between organisms for the passage of phosphorylated compounds across the cell membrane.

Further indication of the importance of phosphorylated derivatives of B_6 comes from the finding [Hendlin *et al.* (41)] that pyridoxamine phosphate and pyridoxal phosphate replace the tomato juice factor activity of several crude materials for *L. lactis*, *L. helveticus*, and *L. acidophilus;* pyridoxamine phosphate was 3 to 20 times as active as pyridoxal phosphate. Pyridoxal, pyridoxamine, pyridoxine, and D-alanine were effective only in much greater concentration.

Strauss (42) has made the interesting observation that growth of certain pH sensitive mutants of *Neurospora crassa* in the absence of vitamin B_6 depends upon the pH being above 6 and the presence of a threshold concentration of free ammonia. Further, that in the absence of vitamin B_6, but with ammonia present, the pH sensitive mutants contain less vitamin B_6 than wild-type strains. Although growth of the Neurospora mutant No. 44602 was inhibited by methionine, added vitamin B_6, ammonia, or threonine overcame this inhibition.

Folic Acid and the Citrovorum Factor

The importance of folic acid (pteroylglutamic acid, PGA), the fermentation *L. casei* factor (pteroyltriglutamate), and the folic acid conjugate present in yeast (pteroyl heptaglutamate) in the nutrition of both animals and microorganisms has been well established. That there are differences in the ability of organisms to utilize the various members of the folic acid group is indicated by the respective activities of these compounds as growth factors for various lactic acid bacteria. While both *L. casei* and *S. faecalis* respond equally to folic acid and not at all to the pteroylheptaglutamate, the growth response of *L. casei* is complicated by the sensitivity of this organism to alanine (43) and various other amino acids (44), small concentrations of thymine (45), and peptides (46). *S. faecalis*, while not as greatly affected by the aforementioned substances, does respond equally to folic acid and to rhizopterin (which contains no glutamic acid), and the presence of this substance in natural materials makes it difficult to evaluate the biologically active folic acid content of such materials (47). Pteroyltriglutamate is only about 6 per cent as active for *S. faecalis* as it is for *L. casei*, for which it is fully active (48).

It is not possible in this review to discuss the development and use of folic acid antagonists and their particular role in inhibiting the growth of both bacteria and animals. A summary of this work has been tabulated [Snell & Wright (49)].

Toennies *et al.* (50) have reported on the interrelationship between methionine and folic acid in the growth of *L. casei*. It was noted that L-methionine lengthened the lag phase of *L. casei* and that during early growth, the organism accumulates folic acid to the point of virtual depletion of the medium of this vitamin. After depletion, growth continues at the expense of the stored folic acid. It is suggested that "as an inhibitor, methionine acts by interfering with the assimilation of folacin, and as a stimulator by serving as a protein building block, sparing cystine."

Another recently established relationship is that between folic acid and and the relatively new bacterial growth factor, the *Leuconostoc citrovorum* factor (CF). An attempt by Sauberlich & Baumann (51) to grow *L. citrovorum* on a synthetic medium failed unless minute quantities of purified liver extracts were added to the medium. Although vitamin B_{12} could not replace the unknown factor, thymidine or folic acid did provide some growth activity, much less, however, than the liver extract [Snell *et al.* (52)].

Through the use of the folic acid antagonist, 4-aminopteroylglutamic acid, it was shown by Sauberlich (53) that concentrates of CF counteracted the toxicity of the antagonist for *L. citrovorum*. Thymidine and folic acid at high levels also reversed the toxicity of the folic acid analogue. Vitamin B_{12} had no effect.

Other studies by Bond *et al.* (54) on the nature of materials related to folic acid disclosed the natural occurrence of substances that were consider-

ably more effective than folic acid in counteracting the antagonisms of methyl folic acid for *L. casei.* This group of substances was called the "folinic acids." Bardos and co-workers (55) soon noted that concentrates of one of the "folinic acids" proved highly effective in promoting growth of *L. citrovorum.* Further, mild acid treatment of the material resulted in the loss of CF, but not folic acid activity.

Recently, Shive *et al.* (56) synthesized a substance by the reduction of formyl folic acid over a platinum catalyst in the presence of ascorbic acid which, after autoclaving in water, exhibited marked CF activity. An amount of the synthetic material equivalent to 0.01 to 0.05 mμg. per ml. of folic acid resulted in half maximum growth of *L. citrovorum.*

The fact that CF is more active than folic acid, not only for the growth of *L. citrovorum,* but in counteracting the inhibition of folic acid analogues, would indicate that it, rather than folic acid, is the enzymatically active form of the vitamin.

May *et al.* (57) have recently disclosed that the elemental analysis of the synthetic factor, folinic acid S-F, corresponds to that of a formyltetrahydrofolic acid. The compound 5-formyl-5,6,7,8,-tetrahydropteroylglutamic acid has been synthesized by Pohland *et al.* (58) and found to have folinic acid or citrovorum factor activity.

Vitamin B_{12}

The research which culminated in the identification of vitamin B_{12} with the anti-pernicious anemia factor in liver is perhaps one of the finest examples of the importance of correlated effort in both bacterial and animal nutrition. It is an interesting paradox that the animal protein factor, associated with various animal but not vegetable proteins, has been shown to be identical with vitamin B_{12} derived from liver (59, 60), and this in turn is identical with vitamin B_{12} produced by microorganisms, which provide the most practical source of this vitamin.

Although the structure of vitamin B_{12} remains unknown, information so far available [Brink *et al.* (61)] indicates a composition of $C_{61-64}H_{86-92}N_{14}O_{13}PCo$. The presence of cobalt at a level of 4.5 per cent is indicative of a minimal molecular weight of about 1,300. Acid hydrolysis of vitamin B_{12} gives a product identified as 5,6-dimethylbenzimidazole, which is similar to a fraction of the riboflavin molecule (62). Vitamin B_{12} (cyano-cobalamin) can be converted to vitamin B_{12a} (hydroxo-cobalamin) by catalytic hydrogenation in the presense of platinum oxide (63). It has been shown that vitamin B_{12} is a cyano-complex of cobalt which can be converted to B_{12a} by the removal of the cyano group. Treatment with cyanide reconverts vitamin B_{12a} to vitamin B_{12} (64, 65, 66). A red crystalline substance has been isolated by Pierce *et al.* (67) from *Streptomyces aureofaciens* fermentations and called vitamin B_{12b}. Hendlin & Soars (68) have shown that vitamin B_{12b} as assayed by *L. lactis* and *L. leichmanii* appears to be identical with B_{12a}. Further evidence for the identity of B_{12a} and B_{12b} was shown by Brockman *et al.* (69)

when vitamin B_{12} was first catalytically reduced to form B_{12a}, after which it was identified with the vitamin B_{12b} prepared by silicic acid chromatography of liver extract.

Because of the influence of numerous substances on the growth of lactic acid bacteria in the absence of added B_{12}, the use of these organisms for the assay of this vitamin and a study of its role in bacterial nutrition has been hampered. For example, thymidine has been shown to replace vitamin B_{12} for numerous organisms (70, 71, 72). Further, the addition of reducing agents such as ascorbic acid is capable of eliminating both the B_{12} and thymidine requirement for *L. lactis* (70).

Kitay *et al.* (73), in an extensive study on the nutritional requirements of a large number of lactic acid bacteria grown in a medium complete with respect to synthetic vitamins, amino acids, and supplemented with tomato juice and enzyme hydrolyzed casein, found that all organisms tested needed thymidine for growth. Other desoxyribosides or high levels of desoxyribonucleic acid could replace thymidine. Although vitamin B_{12} replaced thymidine for most organisms, *L. delbrueckii* 730 and *L. acidophilus* 204 are examples of bacteria that grew upon the addition of thymidine but not vitamin B_{12}. Reducing agents such as ascorbic acid or thioglycolic acid replaced the thymidine or vitamin B_{12} requirement for many organisms in the presence of, but not in the absence of an enzymatic digest of casein in the medium. While the requirement for desoxyribosides was generally uniform for the bacteria studied, a considerable variation was noted in the vitamin B_{12} requirement. It was also shown that the growth-promoting activity of vitamin B_{12a} but not vitamin B_{12} for *L. leichmanii* is largely destroyed when the medium is autoclaved.

The destruction of B_{12a} by autoclaving is prevented by the addition of adequate levels of reducing agents such as ascorbic acid (68). The loss of vitamin B_{12a} during autoclaving is not evident when it is assayed with *L. lactis;* in fact, the activity of B_{12a} for this organism is increased by autoclaving and the addition of reducing substances.

Although the interchangeability of reducing agents, desoxyribosides, and vitamin B_{12} remains unexplained, it has been postulated (72) that vitamin B_{12} may be an essential catalyst in the formation of desoxyribosides. The role of reducing agents has been related by Welch & Wilson (74) to their ability to reactivate inactive oxidized forms of vitamin B_{12}. Despite the attractiveness of such hypotheses, the final explanation of the observed interrelationships awaits further study.

Increasing evidence, particularly in the field of animal nutrition, indicates that vitamin B_{12} plays a role in the metabolism of methionine. Strong evidence for such a role is provided by the observation of Shive (75) that methionine and vitamin B_{12} function interchangeably in allowing growth of *E. coli* in a medium containing sulfonamides.

As a result of a study by Davis & Mingioli (76) with *E. coli* mutants which respond to either methionine or vitamin B_{12}, it may be postulated

that vitamin B_{12} functions in a coenzyme participating in the formation or transfer of labile methyl groups. Mutants which respond to either vitamin B_{12} or methionine do not respond to homocysteine, and furthermore mutants which respond to homocysteine do not respond to vitamin B_{12}. In contrast to lactic acid bacteria that require B_{12}, mutants of *E. coli* requiring this vitamin do not respond to thymidine, and it is suggested that the mutants are stable enough to be useful for the assay of vitamin B_{12}.

The importance of vitamin B_{12} synthesis by the intestinal microflora to the economy of animals is indicated by the report of Halbrook *et al.* (77) that out of 142 isolated strains of microorganisms from poultry house litter only 2.8 per cent did not produce B_{12}, while 42 per cent produced more than 0.4 mμg. per ml. of broth. One organism tentatively identified as a strain of *A. aerogenes* produced more of the vitamin than any of the reference standards (tentative U.S.P.).

Miscellaneous Growth Factors

Wright *et al.* (78) have shown that orotic acid (4-carboxyuracil), at a level of 10 to 100 μg. per tube, replaces the need for large amounts of a natural material for *L. bulgaricus*. Orotic acid and 4-carboxyuridine have been suggested as precursors in the biosynthesis of uridine and/or cytidine, and 4-carboxyuridine or some related derivative or orotic acid may be the uncharacterized acid-labile factor in natural materials.

Guirard *et al.* (79) have reported an unidentified water-soluble factor that replaces the growth-promoting action of acetate for *L. casei*. This factor was much more active in crude form than acetate. Concentrates of the pyruvate-oxidation factor and of protogen also showed growth-promoting activity for *L. casei* [Snell & Broquist (80)]. As a result of these observations, it was concluded that protogen, the pyruvate oxidation factor, and the acetate-replacing factor for *L. casei* are probably identical.

In this connection it has been reported by Lytle & O'Kane (81) that *Streptococcus cremoris* requires acetate, sorbitan mono-oleate, or reticulogen for growth in a synthetic medium. These observations suggested that the pyruvate oxidation factor (POF) might be the active fraction of reticulogen, since POF and the factor that replaces oleate or acetate in the nutrition of certain lactic acid bacteria are probably identical. Reticulogen is known to be a good source of POF.

A factor found in a tomato juice filtrate treated with norite and a phenol n-butyl alcohol extract of injectable liver extract has been reported by Peeler & Norris (82). The presence of the unidentified factor was demonstrated by the use of a very light inoculum and a short incubation time. The factor was found concentrated in liver extracts, fish, meat, yeast, and tomato juice; dried whey was a relatively poor source.

It has been shown that the activity for *L. lactis* Dorner of the tomato juice factor concentrate obtained from *Streptomyces griseus* fermentation is due to its pyridoxamine phosphate content (41).

Amino Acids and Peptides

Although this review pays little attention to the vast amount of work that has been directed toward establishing the amino acid requirements of numerous bacteria and the use of microorganisms for the determination of amino acids in natural materials, these contributions should not be minimized and should be studied carefully by those interested in nutrition.

Two general areas of investigation will be cited to illustrate the use of bacteria in the study of the biochemical role of amino acids and peptides. A series of reports have been made of the utilization of D-amino acids by lactic acid bacteria. Camien & Dunn have shown that the utilization of D-glutamic acid (83) and D-methionine (84) by *L. arabinosus* is strongly inhibited by relatively high concentrations of aspartic acid and methionine, respectively. Leucine and valine in moderate concentrations interfere with the utilization of D-isoleucine by *L. arabinosus* (85, 86). Because of these observations, it has been suggested (84) that "the greater reduction by specific amino acid inhibitors of D-amino acid activity than of L-amino acid activity for lactic acid bacteria probably is a general phenomenon."

A fruitful series of experiments on the utilization of various amino acids and their derivatives, using artificially produced mutants of *E. coli*, has been reported by Simmonds *et al.* Experiments have been done with phenylalanine, tyrosine (87), leucine (88), proline (89), and methionine [Taylor *et al.* (90)]. Of particular interest is the fact that peptides of proline are more active than the amino acid itself in promoting growth of the prolineless *E. coli* mutant.

The exact role of peptides in the nutrition of bacteria remains obscure. Additional evidence is presented by Kitay & Snell (91) that one or more factors present in charcoal-treated enzymatic digests of casein are essential for the growth of several cultures of *L. bulgaricus*, *L. helveticus*, and *L. lactis* and stimulate growth of many more lactic acid bacteria.

The Effect of Fatty Acids on Growth of Bacteria

Certain long-chain unsaturated fatty acids in small quantities serve as growth factors for some organisms, but are inhibitory when used at higher concentrations. Some common saturated fatty acids appear not to be active in these respects (92, 93). Among the unsaturated acids, oleic and linoleic acids were found to be stimulatory for strains of *Corynebacterium diphtheriae* (94), *Clostridium tetani* (95), *Clostridium sporogenes* in the absence of biotin (96), *Mycobacterium tuberculosis* in the presence of Tween 80 or albumin (97, 98), *Erysipelothrix rheusiopathiae* with the addition of saponin (99), some lactic acid bacteria usually in the absence of biotin or in the presence of Tween 40 (92, 100, 101, 102), and the fungus, *Pityrosporum ovale* (102). The amounts of the acid required as a growth factor are usually small, e.g., 0.2 to 1.0 μg. per ml. in the case of lactic acid bacteria. In an extensive study by Pollock *et al.* (93), it was observed that for an unidentified diphtheria bacillus Q,

at least one double bond appears to be absolutely essential, but its exact position is less important. The configuration around the double bond is apparently not critical. . . . A single hydroxyl group, however, in the 11 position (ricinoleic acid), abolishes activity. Shortening the chain length by two methyl groups, leaving the double bond in the same position relative to the carboxyl group (palmitoleic acid), does not greatly reduce the activity, but the C 22 acids, erucic and brassidic, with the double bond in the 12–13 position, are quite inactive.

It was also suggested that the acids were incorporated into the bacterial cell "to form some essential constituents of the cell protoplasm" because of "the linear relationship between growth and fatty acid concentration of the medium."

On the other hand, the same long-chain unsaturated fatty acids can be detrimental to the same bacteria and to a number of others. The amounts required need not be much larger than those capable of stimulating good growth of the organisms. Thus, for example, fatty acids at a concentration of 2 μg. per ml. will inhibit the growth of certain lactic acid bacteria [Williams *et al.* (92)]. It has also been shown by Burtenshaw (103) that unsaturated fatty acids are the main components of the secretions of skin which have high bactericidal effects on hemolytic streptococci, diphtheria bacilli, and pneumococci. Sodium oleate has been utilized by Avery (104) as the selective agent in a medium for the cultivation of *Hemophilus influenzae*. The growth of streptococci and pneumococci in this medium was suppressed. *Hemophilus pertussis*, however, was found by Pollock (105) to be sensitive to small amounts of oleic acid. Inhibitory effects of the long-chain unsaturated fatty acids could be removed and the acids made stimulatory by the addition of serum, albumin, starch, activated carbon, or Tween 40 along with saponin. While in the absence of albumin the inhibitory concentration of oleic acid for *M. tuberculosis* is 1.0 μg. per ml., in the presence of albumin, oleic acid stimulates the growth of the bacillus at concentrations up to 100 μg. per ml. [Dubos (98)]. It was suggested that the fatty acids are adsorbed on the accompanying materials and then dissociated gradually in minute quantities for utilization by the bacteria (105).

Complex organic media were found to be antisporulatory to *Bacillus larvae* and could be rendered sporogenic by treatment with starch or activated carbon. The inhibitory factor was identified as a saturated fatty acid of intermediate chain-length, such as capric, lauric, tridecylic, or myristic acid (106, 107). Foster & Wynne (108) have shown, however, that the germination of *Clostridium botulinum* is inhibited by the unsaturated C18 fatty acids.

A synergistic effect of saturated and unsaturated fatty acids has been observed on growth of certain lactobacilli by Hassinen *et al.* (109).

When *L. casei* is grown in the presence of oleic acid, a disparity was observed by Williams & Andrews (110) between the turbidimetric and titrimetric results, suggesting an interference with normal glycolysis "so that only a portion of the normal amount of the acid is produced whereas the cell growth is unhampered".

Utilization of Carbon Sources

Although systematic research in this specific field has been limited, the following observations are of interest. The growth of *Eberthella typhosa* was found by Barkulis (111) to be inhibited to some extent by L-rhamnose, L-arabinose, L-sorbose, and L-fructose, but the D-isomers of these carbohydrates were not inhibitory. Noninhibited mutants are produced when the parent strains are cultivated in the presence of the L-compounds. Despite the inhibitory effects observed, some growth of the organisms did occur, even though the L- and D-forms of the sugars were not attacked or utilized as far as could be determined.

In the oxidation of glycerol by *S. faecalis*, VanDemark (112) found that thiamin, niacin, riboflavin and an unidentified factor were needed as coenzymes. Pantothenic acid, biotin, and folic acid may be required to a limited degree, while pyridoxamine, vitamin B_{12} and *p*-aminobenzoic acid are not essential for glycerol oxidation. Forsyth & Webley (113) found that in the synthesis of polysaccharides from sucrose by bacteria, two reducing sugars, glucose and fructose, are liberated. The amounts of the reducing sugars varied with the bacteria and the type of polysaccharide synthesized. The investigators suggested an involvement of both polymerizing and hydrolytic enzymes. Their results could also be explained by the type of mechanism concerned in the decomposition of maltose into glucose and polysaccharide by the action of amylomaltase [Doudoroff *et al.* (114)].

It is also interesting to note that heparin, a muco-polysaccharide, has a bacteria-inhibiting effect in the presence of pus or "trauma blood." This reaction is not evident *in vitro*, even at a concentration of 100,000 p.p.m. or above (115).

Although virulent strains of *H. pertussis* required and utilized 20 per cent of starch for growth, avirulent strains did not (116, 117). When virulent organisms were suspended in phosphate buffer containing starch the same amount (20 per cent) of the carbohydrate disappeared. One is led to think that starch was not metabolized by the organism but also acted by adsorbing inhibitory materials. This would lend some support to the contention (93) that starch stimulates the growth of the organism by removing the inhibitory effects of long-chain unsaturated fatty acid, possibly present as a contaminant of the media, on virulent but not on avirulent organisms. Since gums and inorganic adsorbents supported little growth of *H. pertussis*, some special mechanism must be involved that permits charcoal and amylose to replace starch for growth of the organism. A "simplified liquid culture medium" has been prepared for *H. pertussis* which supports good growth [Verwey *et al.* (118)]. However, the addition of 1.5 to 2 per cent agar in the liquid medium necessitates the incorporation of 10 to 20 per cent of human blood in order that adequate growth be attained. It is possible that the batch of agar used might have contained enough fatty acids to have caused an adverse effect (119).

Antibiotics and Antimetabolites

It is a common observation that the intestinal flora is altered as a result of the administration of many antibiotics. This is attributed to the suppression of susceptible organisms in the intestine. There still exists a possibility that the growth of resistant organisms may be stimulated to some extent by this group of substances. Some bacteria are known to require antibiotics for growth (120, 121, 122).

The analogues of growth factors have been used extensively to study the nutrition and metabolism of bacteria, and they have been used or proposed for use as chemotherapeutic agents. Importance has been attached to the physical chemical properties of such compounds, for example, the electropolarity and the number of hydrogen bonds, instead of the mere replacement of certain radicals or groups in these compounds (123). Of great interest are those chemicals having chelating activities (124). A full discussion on this subject is beyond the scope of this review. The excellent collection of short discussions on growth factor analogues led by Fildes (125) should be consulted for this purpose.

Maass & Johnson (126) found penicillin to be firmly bound by a catalyst-like component of the bacterial cell which led to the inhibition of bacterial growth. The binding was so firm that bound radioactive penicillin did not exchange even with high concentrations of free penicillin. It seems that resynthesis of this catalyst-like component of the cell may be the chief prerequisite for cell division.

Some Aspects of Fungal Nutrition

Knowledge concerning the nutrition of fungi, particularly the pathogenic species, is rather meager. Wolf (127) recently made an extensive study on molds of meat. It dealt mainly with the suppression of the spoilage of meat. For wood-rotting fungi, it was found by Jennison (128) that no nutrilites other than thiamine or biotin appear to be essential for their continued growth and that no other compounds can replace these two vitamins. Thiamine and biotin are qualitatively interchangeable. Addition of glutamate, asparagine, purines, or pyrimidines stimulated growth in the presence of the above two substances. The combination of folic acid and/or inositol with thiamine appears to have an inhibitory effect. In general, organic sources of nitrogen gave better growth than inorganic nitrogen compounds. The only sulfur-containing amino acids examined, cysteine and methionine, supported poorer growth than nonsulfur-containing amino acids. Sugars usually cause a marked increase in acidity of the media. The size of the pellets of growth appeared to be correlated with the type of nutrient instead of with the species of the organism.

Of increasing interest to the clinician and to the mycologist are the patients whose death can be attributed to a fungal infection during therapy for bacterial infections with antibiotics (129, 130). A number of similar

observations have been made recently in the Grace-New Haven Community Hospital, New Haven, Connecticut. These reports tend to suggest a close relationship of antibiotics to these mycotic infections. The stimulatory effect of streptomycin on the *in vitro* growth of certain fungi (131) and an increased mortality with penicillin therapy of chick embryos and rabbits infected with candida (122) have been reported. The exact mechanism of such effects remains to be explained. A factor that must be considered, aside from the direct effects on the intestine and microflora, is that the antibiotics may cause retardation of the growth of tissues and so alter their metabolism that their resistance to fungal infestions is lowered.

Mineral Requirements of Bacteria and Fungi

An extensive study has been made by Webb (132, 133, 134) of the effects of magnesium on cell division. It was noted that the ion is essential for the normal division of cells. Both a deficiency and an excess of the element led to the formation of filamentous cells of bacilli, while the division of chromatinic bodies was not appreciably interfered with. It seems that magnesium is needed for the separation of daughter cells of bacilli. For cocci, its excess or deficiency mainly influences their growth, but not their morphology. "The magnesium requirements of Gram-positive bacteria, are considerably (some ten times) greater than those of Gram-negative bacteria, possibly because the former incorporate magnesium into the structure of the Gram complex." There are indications that magnesium is involved in the synthesis of bacterial protoplasm. Magnesium also stimulates nitratase adaptation in certain bacterial species (135).

When vitamin B_{12} was added to the medium (136), phosphorus (as P^{32}) uptake by *L. leichmanii* was increased, as evidenced particularly by its marked increase in desoxyribonucleic acid (about 4 times as much as in the controls). It has been noted by Bunting *et al.* (137) that *Serratia marcescens* produced demonstrable amounts of polysaccharide at low concentrations of phosphorus. The minimal phosphorus requirement of Group A hemolytic streptococci appears to be small and of the order of 0.09 μg. per ml. in an otherwise phosphorus-free semi-synthetic medium (138). The organic nitrogen source of this medium was an enzyme hydrolysate of lactalbumin. It is pertinent to note that acid-hydrolyzed lactalbumin or a mixture of amino acids failed to support growth of this organism and, apparently, unidentified factor(s), probably peptidic in nature, are present in enzymatically hydrolyzed lactalbumin.

It has been suggested by Brodie *et al.* (139) that one of the manifestations of the physiological difference between smooth and rough variants of a single culture is the greater permeability of rough variants to electrolytes in the presence of bile salts.

A linear increase in the total population of a culture of *Bacterium lactis aerogenes* has been shown by Eddy *et al.* (140) to accompany the increase in the potassium content of the medium. It was postulated that potassium is an enzyme-activator.

An extensive investigation has been reported by MacLeod & Snell (141) on the ion antagonism and inorganic nutrition of lactic acid bacteria. It was suggested that manganese acts as an essential enzyme-activator. Its effect could be replaced by other nonessential inorganic ions. The inhibitory action of zinc was due to the formation of an inactive metalloenzyme by the ion and an apoenzyme, which could be activated nonspecifically by manganese, magnesium, calcium, and strontium.

Fragraeus (142) observed that the concentration of sodium chloride influenced the growth of *Staphylococcus aureus*, *E. coli*, and *Proteus vulgaris*. *S. aureus* grew abundantly in serum broth containing 8 to 10 per cent sodium chloride but was inhibited at a concentration of 16 to 18 per cent. *E. coli* and *P. vulgaris* were inhibited by 10 per cent of the salt. Thus, a medium containing 10 to 14 per cent sodium chloride in broth can be used for the preferential isolation of staphylococci when *E. coli* and *P. vulgaris* are present.

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NUTRITION AND METABOLISM OF PROTOZOA

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A review of certain aspects of protozoan nutrition and metabolism appeared in this publication in 1947 (68), which together with earlier reviews (14, 15, 28, 32, 38, 64 to 67, 71, 83, 107) make it unnecessary for this discussion to include more than the last four years of research reports on most of this material. In none of these reviews, however, have the nutritional problems posed by the two membered cultures, and especially the carnivorous ciliates, been discussed. Therefore a section will be devoted to the work which has been done on two membered cultures, because it is felt that some of the problems presented there are unique. While it is always quite impossible to deal in direct quantitative or qualitative biochemical terms under these conditions, a number of the results are suggestive for future studies. The rest of the review will deal with studies on protozoa in pure culture. It is obvious that interpretations of results of a biochemical nature are largely or completely invalidated for a given microorganism when unknown and uncontrolled associated forms are present. It is only since the establishment of pure (bacteria-free) cultures of protozoa that comparative nutritional reports have taken on real meaning.

During the early days of pure culture work with protozoa, a number of techniques were developed for ridding the protozoan of its associated organisms. These techniques have been reviewed (36). The most successful ones depended on methods of washing away and diluting out the contaminating microorganisms. They were tedious and time-consuming. More recently the use of various antibiotics (31, 77, 80, 89, 109) has proven most useful for this purpose. One needs only to use those types of antibiotics which are well tolerated by the particular protozoan to be sterilized and to use them in diverse enough combinations to prove bacteriostatic to the widest variety of contaminants. The wealth of experimental material within the Phylum Protozoa may now be utilized by the biochemist with the virtual certainty of results which will prove valuable to our knowledge of nutrition and metabolism.

Two Membered Cultures

In order to establish a two membered (*zweigliedrige*) culture, the protozoan must be freed of all extraneous forms. In many types, it has not been possible to obtain growth of the sterilized protozoan in the absence of living food. When this is the case, appropriate food organisms are introduced into the medium, the fluid portion being either nutrient or non-nutrient to the food organism. When the protozoan is a bacteria feeder in nature, some type of bacterium is most frequently used, although this is not always so.

Obligate carnivores must be supplied with the types of protozoan food which they will accept. In a number of species so far investigated, the synthetic mechanisms have degenerated to such an extent that they are incapable of building for themselves certain, as yet unknown, complexes which are present only in living prey. For many types, the prey must not only be alive when ingested, but extremes of selectivity are practiced so that one species of prey may prove acceptable and adequate while a closely allied species will not.

The family Colpodidae contains a number of genera which have been the subject of investigation. The most common genus is *Colpoda*, a holotrichous ciliate living in fresh water ponds where plant infusions make available large numbers of bacteria. This ciliate reproduces by fission while protected by a thin cyst membrane, and when the food supply is low it forms resistant cysts which are impervious to drying. When *Colpoda steinii* [see Burt (8) for specific designation] was freed of its associated bacteria, it was found to be incapable of growth in various nutrient media alone or with dead bacteria (58). Growth was possible when any of a number of species of bacteria was added singly in distilled water, although *Aerobacter cloacae* promoted most rapid and luxuriant growth. One species of *Flavobacterium* was not ingested while two pigmented species, *Chromobacterium violaceum* and *Serratia marcescens* were lethal to the ciliate. It was found that the toxicity of the pigmented forms was due to the pigment itself, for the ciliate was able to utilize *S. marcescens* for food when in its non-pigmented phase. Moreover, it was shown that, under certain conditions, acclimatization to the pigment of *S. marcescens* was possible, which probably accounts for the ability of Phelps (79) to culture *Paramecium aurelia* on a pigmented strain.

Later (103, 104) it was found that *Colpoda* could be cultured in the absence of living food provided a bacterial "plasmoptyzate" was added, together with yeast extract. The plasmoptyzate was presumably protein in nature and was heat labile. Its growth promoting power diminished with time. It seems probable that *Colpoda* represents a fairly non-selective organism which has become dependent through enzyme loss upon other cells for certain complexes of a labile nature. The heat stable yeast factors could be replaced by thiamine, pantothenic acid, pyridoxine, nicotinamide, and riboflavin (21, 102). It is probable that these growth factors are not the only known compounds required, as the "plasmoptyzate" was admittedly not vitamin free.

Employing *Aerobacter* as the food organism, Kidder & Stuart (59) showed that the most important factor leading to encystment (resistant cyst formation) in *C. steinii* was lack of food. Encystment was shown not to result from crowding, as had been commonly supposed when raw cultures were studied, for when no deleterious bacteria were present and the food organism was offered in large amounts (hanging drop culture), no encystment occurred even when the ciliate had reproduced to a concentration leading to physical contact one with the other.

More recently, Garnjobst (20) has shown that not only total lack of food but certain nutritional deficiencies will bring on encystment in *Colpoda*. Thus she was able to induce encystment by omitting thiamine, pyridoxine, pantothenic acid or nicotinamide, or by destroying the heat labile "plasmoptyzate" factor.

Selectivity to a marked degree [see (36) for account of extreme selectivity in *Tillina canalifera*] is rare among the bacteria-feeders (7), although distinct differences of nutritive value are often noted (58). Among the carnivorous forms which have been investigated in two membered cultures, however, strict selectivity is often found. Thus, in another genus of the Colpodidae, *Bresslaua*, Claff, Dewey & Kidder (11) found that *B. vorax*, *B. insidiatrix*, and *B. sicaria* would all ingest *C. steinii* and normal growth would result. *B. sicaria*, however, refused to ingest *Glaucoma scintillans* while for the other two species, this food organism was adequate. On the other hand, *Tetrahymena geleii* was accepted by all three species of *Bresslaua* but was quickly lethal to *B. insidiatrix* and *B. sicaria*, and promoted only poor growth of *B. vorax*. Of these three species, only *B. insidiatrix* was dependent on moving prey to evoke the ingestion response, and for it only the ciliate *Colpoda* proved adequate nutritionally.

Dewey & Kidder (12) succeeded in establishing *Perispira ovum* in double culture with *Euglena gracilis*. No conclusions could be reached regarding its nutritional requirements other than its extreme food selectivity. This holotrichous ciliate, a member of the family Spathidiidae, possesses a mouth which is closed except when stimulated by appropriate food organisms. These food organisms must be euglenoid flagellates and only contact of the flagellum with the mouth ridge evokes the swallowing response. It was impossible to determine whether this stimulus should be classed as chemical or physical, or both.

The suctoria are sessile organisms possessing tentacles for the capture and ingestion of prey. Recently, Rudzinska (84) has shown that *Tokophrya infusionum* can be grown in a two membered culture with *Tetrahymena* as food. She showed that there was an optimal number of food organisms ingested for optimal reproduction (production of ciliated embryos), the number of embryos produced diminishing to zero in starved or overfed suctorians. Longevity of individuals, on the other hand, was found to be decreased as food increased.

Entozoic amoeba have been cultured for many years in the presence of living bacteria. Inasmuch as *Endamoeba histolytica* will grow aseptically in liver abscesses, it appears likely that the "living factor" which they are incapable of synthesizing for themselves is being supplied by the living bacteria in cultures and by the tissue cells in the abscess. Recently, Griffin and his associates (24 to 27) have again shown that living bacteria alone are not nutritionally adequate for *Endamoeba terrapinae* and *E. histolytica*, and that growth depends upon the inclusion of rice starch of particular granule size, and factors from serum. They were able to replace the serum with

cholesterol and much more adequately with cholesterol and oleic acid. Recently, Phillips (81) has been able to obtain cultures of *Endamoeba* by replacing the bacteria with *Trypanosoma cruzi*.

One of the first to successfully grow a hypotrichous ciliate in two membered cultures was Provasoli (82). He showed that *Kahlia acrobates* would multiply on the flagellates *Polytoma caudatum*, and *P. uvella* in meat peptone, but not on two strains of *Polytomella agilis*. No growth occurred when living flagellates were replaced by flagellates heated 9 min. at 44°C., macerated fresh liver, liver extract plus dead yeast plus fresh rabbit kidney [see Glaser & Coria (23)] or heat killed bacteria (three strains).

Perhaps the most informative work on the nutrition of hypotrichous ciliates is that of Lilly (61). He was able to show that *Stylonychia pustulata* and *Pleurotricha lanceolata*, normally quite omnivorous in nature, require, first of all, living prey. Establishment in two membered cultures was achieved by use of watery extracts of timothy hay and any one of the holotrichous ciliates *Tetrahymena geleii*, *T. vorax*, and *Colpidium campylum* and the flagellates *Euglena gracilis* and *Astasia klebsii*. Under these same conditions, however, neither the ciliate *Glaucoma scintillans* nor the flagellate *Chilomonas paramecium* was nutritionally adequate, although both were ingested. Living yeast (suspended in distilled water) was nutritionally adequate for *S. pustulata* but not for *P. lanceolata*.

Lilly showed that the food organisms became nutritionally inadequate when they were killed by sudden exposure to a temperature of 37°C. or by freezing or by altering the osmotic pressure by salt increase. None of these methods would be expected to denature protein per se but might conceivably alter enzyme systems in the food organisms. This type of requirement appears, then, to be widespread and offers interesting possibilities for future investigations of what may turn out to be enzyme incorporation by the carnivorous or herbivorous microorganism.

Of as great interest from a biochemical standpoint, and certainly more amenable to solution, was the finding by Lilly that the two hypotrichs mentioned above required supplementary growth factors that were not supplied in the protoplasm of the prey or were supplied in insufficient amounts. Neither *S. pustulata* nor *P. lanceolata* could be grown in the presence of food organisms (*Tetrahymena*) unless some factor (or factors) of plant origin was present. Effective concentrations of the supplementary factor were found in yeast and various higher plants. It was found to be heat stable, soluble in water and 70 per cent alcohol, and was adsorbed on charcoal and Fuller's earth. Recently Lilly and his associates (62) have reported that a third hypotrichous ciliate, *Euplotes patella* is more exacting still. It requires live food (*Chilomonas paramecium* is best), a factor or factors from yeast (presumably the same as are required by *S. pustulata* and *P. lanceolata*), a factor from liver, and a factor from killed bacteria (strain undesignated). Omission of any one of these factors resulted in immediate nutritional failure.

It would appear that the highly specialized hypotrichous ciliates offer excellent experimental material for future work on unidentified growth factors. It has been found in the past that a new growth factor, discovered through the loss of ability of any organism to carry out its synthesis, will almost certainly prove significant in our understanding of metabolic reactions in general. One needs only look at the historical development of pteroylglutamic acid and vitamin B_{12} to realize the significance of microorganisms as guides.

Pure Cultures

The term "pure culture" is used here in its broader sense. It simply means that the organisms under consideration are cultured in the absence of associated living types. They are bacteria- and mold-free. It does not necessarily imply that the medium upon which they grow is chemically defined. It is, of course, true that this type of culture offers many advantages over the two-membered type, and from the former we may confidently look for rapid advances in our knowledge of nutritionally important chemical components and their metabolic functions.

Plant Flagellates

Extensive reviews of the excellent work which has been done on the nutrition and metabolism of the plant flagellates have been cited (14, 15, 60, 67, 68, 71). During the past few years, several important papers have been published dealing with green and colorless plant flagellates. These flagellates, unlike the rest of the members of the Phylum Protozoa, possess remarkable synthetic capacities. This group has retained, in common with most members of the plant kingdom, synthetic enzymes for the elaboration of most of the complex organic molecules needed for metabolism.

Even where high synthetic abilities occur, however, it sometimes can be shown that pre-formed metabolites are stimulatory. Thus, with a strain of *Chilomonas paramecium* that can grow indefinitely in an entirely inorganic medium (72 to 76), Pace (78) found that growth was accelerated when thiamine, riboflavin, nicotinic acid, and pyridoxine were added in the presence of iron. Under similar conditions, pantothenate had no accelerating effect.

Inorganic requirements of plant flagellates have received attention and an excellent review of techniques, especially the use of chelating agents for the study of trace elements has been presented by Hutner and his associates (29).

Recently, Hutner *et al.* (30) have shown that *Euglena gracilis* var. *bacillaris* is incapable of synthesizing vitamin B_{12} and thiamine. On a basal medium containing inorganic constituents, plus thiamine, no growth resulted unless vitamin B_{12} was added. Regular growth response was obtained to increments of vitamin B_{12} and it was found that half maximal growth resulted upon the addition of 0.01 mμg. of the crystalline vitamin. It was found

possible to use *Euglena* for assaying vitamin B_{12} in crude materials, and it would appear that this type of microorganism might surplant bacteria for this purpose inasmuch as the flagellate responds specifically to vitamin B_{12} and does not respond to thymidine. It is interesting to note that *Astasia klebsii*, a close relative of *Euglena*, although colorless, also requires thiamine and vitamin B_{12} and the quantitative responses of the two flagellates are identical (105).

Lwoff *et al.* (69) have considered again the question of the acetate requirements of the plant flagellates. It has long been known that glucose will not satisfy the carbon requirement of many types, including *Polytomella caeca*. Using this colorless flagellate, Lwoff, Ionesco & Gutmann (69) have identified glucose-1-phosphate, which could not have been formed from glucose due to a lack of hexokinase. They suggest that hexophosphates are formed from phosphorylated trioses. Thayer (105) found that *A. klebsii* could use starch and glucose-1-phosphate but not glucose. This could also be best explained by the lack of hexokinase. A number of well-known phosphorylated compounds have been identified in *Euglena* (1).

Parasitic Flagellates

The exact nature of the nutritional requirements of parasitic flagellates is still largely obscure (see earlier reviews). In no case have chemically defined media permitted growth of these forms. The nearest approach appears to be the report of Weiss & Ball (110) on the amino acid requirement of *Trichomonas foetus*. Using the medium of Kidder & Dewey (38a), they found, by an omission technique, that this flagellate was incapable of synthesizing arginine, glycine, tryptophan, histidine, isoleucine, leucine, lysine, threonine, methionine, phenylalanine, proline, serine, and valine. One recognizes at once the similarity between these amino acids and those required by the growing rat. In addition to the ten amino acids essential for the rat, glycine, proline, and serine are required by *T. foetus*. Neither free amino acids nor whole proteins promoted optimum growth of *T. foetus*. Best growth was obtained with added serum or enzymatically treated proteins. Weiss & Ball suggest the possibility of a partial strepogenin (111, 112) requirement.

A report of interest on *T. foetus* has been made by Ford & Morgan (17) in which they show that the inclusion of *Vibrio fetus* in the crude medium employed greatly increased the growth of the flagellate. They believe this increase may be accounted for by the release of nutrients by *Vibrio* for use by the flagellate. Increasing the carbon dioxide content of the medium also increased flagellate growth. They believe that this may be due to increased buffering (cultures rapidly become alkaline) or a tendency to force more anaerobiosis, or both.

Trichomonas vaginalis has been grown for some time in crude medium with the addition of serum. More recently, Sprince & Kupferberg (98) have obtained good growth using pancreatic digest of casein to which was added

the B-vitamins, adenine, guanine, uracil, acetate, ribose, asparagine, and filtered human serum. The above medium was formulated for future work on the serum factors. They later (99) used it (minus serum) as the basal medium with which they showed that the serum could be fractionated into ether-insoluble and ether-soluble parts. The ether-soluble fraction was dialyzable and heat stable and could be replaced by linoleic acid. The ether-insoluble fraction could be replaced by serum albumin. That other factors essential for growth of the flagellate were present in both fractions was shown by the failure of the medium to support growth when only known lipids and fatty acids (including linoleic acid) were added together with human serum albumin. Sprince (97) later reported that the lipid fraction was protein free and the ether-insoluble fraction activity resided in the serum proteins precipitable by 50 per cent ethanol.

Sampath & Little (101) have reported the culturing of *Trypanosoma cruzi* on a peptone-hydrolyzed casein, glucose medium to which is added sodium chloride, β-alanine, glycine, pteroylglutamic acid, niacinamide, thiamine, choline, and fresh or dehydrated coagulum of rabbit red blood cells. All components of this medium can be sterilized by autoclaving.

Lwoff & Valentini (70) have reported the aseptic culture of *Cepedea dimidiata* from the rectum of *Rana esculenta*. The Opalinids have, until recently, been classified with the astomatous ciliates, but it is generally becoming accepted that they are in reality Hypermastigote flagellates. The medium used was very complex containing gum arabic, yeast extract, ascorbic acid or cysteine, peptone, and frog liver.

Sporozoa

Mention should be made of the report of Ball and his associates (2, 3) on the culture of the malarial organism *Plasmodium knowlesi*. In a complicated system requiring at first whole blood and later whole red blood cells, they were able to show an apparent requirement for glucose and *p*-aminobenzoic acid. Stimulation resulted from the addition of a number of amino acids, purines, and pyrimidines and certain vitamins, provided they were all added together. The culturing of any intracellular parasite offers almost insurmountable difficulties and the prospects of critical nutritional information regarding this group seem remote at present. An entirely different approach to the nutritional problem of the sporozoa has been used by Becker and his associates (4, 5, 6). They have varied the vitamin content of the food of the host (rat) and noted the effect on the oöcyst production of the parasite (the coccidium *Eimeria nieschulzi*). A complete discussion of their results, while of general interest, is out of place here.

Ciliates

During the period covered by this review, by far the greatest amount of work, in the protozoan nutritional field, has been done on the ciliates. Most of this work has been done with the holotrichous ciliate, *Tetrahymena*.

There are a few important reports on other genera that will be considered.

Paramecium.—This ciliate has probably been used as laboratory material longer than any other single protozoan (95). Crude cultures are easy to maintain. Its specialization is such, however, that we are still far from an understanding of its enzymatic potentialities, except that they are limited.

In 1942, Johnson & Baker (34) showed that the heat-labile requirement or "living factor" requirement of *Paramecium multimicronucleatum* could be met by adding juice expressed from yeast cells by pressure. Later, Johnson & Tatum (35) fractionated this pressed yeast juice by dialysis into two parts, both of which were required. The nondialyzable fraction was rendered inactive by a temperature of 55°C. or above and would stand repeated precipitation by ammonium sulfate. The dialyzable fraction was heat stable. They reported growth of *P. multimicronucleatum* in distilled water containing these two fractions, but the addition of proteose-peptone was stimulatory. Recently, Johnson (33) reported in an abstract on the continuation of this work. He found that he can replace the heat-labile fraction with hydrolyzed nucleic acid. Thus, all the components of the medium can now be autoclaved. When this is done, peptone must be included. He was unable to replace the peptone with amino acids, nor could he replace the heat stable, dialyzable yeast juice fraction with known vitamin mixtures. These results may possibly be interpreted as follows: the nondialyzable fraction represented a protein-nucleoprotein complex which became inhibitory upon being heated. Unheated, however, it supplied nearly optimum amounts of amino acids and some other unknown factor (present also in peptone) as well as nucleic acid components. The dialyzable fraction may have contained, in addition to the known vitamins, an unknown metabolite, possibly similar to one of the factors required by the hypotrichs (61, 62). The continuation of this work should prove most interesting and valuable.

P. aurelia is routinely cultured for genetic work in a lettuce infusion culture of live *Aerobacter aerogenes* (96). Recently, van Wagtendonk & Hackett (109) have succeeded in growing this organism in pure-culture. They report that there are three required parts to the medium: (*a*) autoclaved yeast autolyzate of a particular brand (Basamin-Busch—no other brand gave the same result); (*b*) lettuce infusion (autoclaved); (*c*) *A. aerogenes* (24-hour culture, autoclaved). Obviously this is only preliminary work, but it does demonstrate that this important ciliate possesses all the enzymes needed for the synthesis of heat labile compounds.

Colpoda.—The sterile culture of *C. steinii* (*duodenaria*) has been achieved (103, 104) and was described in a previous section.

Glaucoma.—The early work of Kidder (37) seemed to indicate that *Glaucoma scintillans* required particulate food. Growth was obtained only when yeast cell fragments were present in the medium. Recently, however, Fuller (18) has found that growth is not only possible in completely dissolved nutrients, but even in media based on crystalline amino acids. Some informa-

tion was gained concerning its amino acid requirements, when it was found to require proline, glycine, and serine in addition to those essential for the growing rat. Successful growth was limited to those media containing crude materials, such as certain liver fractions or plant leaf extracts. Little is known concerning the vitamin requirements of *G. scintillans*.

Tetrahymena.—During the past four years, a number of papers have been published on the nutrition, metabolism, and enzyme pattern of *Tetrahymena geleii*. Most of this work has been extensively reviewed elsewhere (48), so the present review will be brief, except for certain papers not previously mentioned.

It is possible to obtain excellent and reproducible growth of *T. geleii* in media, the constituents of which (with one exception) are known compounds (13, 43). This animal microorganism does not possess the necessary enzymes for the synthesis of the nine amino acids essential to the adult rat and, like the rat, synthesizes inadequate amounts of arginine (40). When all of the ten amino acids are present in moderate to high concentrations, growth fails unless serine is added (40). Serine appears to remove certain inhibitory effects of otherwise essential amino acids. With one strain of *T. geleii* (strain E), Elliott (16) found serine to be an absolute requirement. By additions of a number of nonessential amino acids, growth of *T. geleii* W is greatly increased (13).

A number of amino acid interrelationships have been reported. Interpreting these from an enzyme point of view, one can make the following statements. Enzymes are present for the oxidation of the *p*-position of phenylalanine to tyrosine, but the reverse reduction is impossible (38); enzymes for the methylation of homocysteine are lacking [even in the presence of labile methyl donors and added vitamin B_{12} (48a)] but the conversion of homocystine to cysteine is possible (22, 41, 48). Methionine cannot be synthesized from any known compound, such as cystathionine (48a).

A number of keto and hydroxy analogues of the natural amino acids have been investigated for replacement or sparing action and so far all have proven to be unavailable to *T. geleii* W (48).

Recently an attempt was made to determine the inorganic requirements of *T. geleii* W (51). It was found that a high requirement for magnesium, potassium, and phosphate exists, but no requirement for calcium could be demonstrated. It had earlier been shown that both copper and iron were required (43). Failure to demonstrate a requirement for any of the trace elements was attributed to probable inorganic contamination of components of the basal medium. It may be possible to investigate this problem further by employing chelating agents (29).

T. geleii W requires, under the usual conditions of culture, the following B-vitamins: thiamine, niacinamide or niacin, pteroylglutamic acid, pyridoxal or pyridoxamine, and protogen (44, 45).

It was shown that pyridoxine was probably inactive and the apparent activity of large amounts came from the spontaneous amination to pyridoxamine. Pyridoxal phosphate, an active co-enzyme in some systems, proved only 75 per cent as active to *T. geleii* W as either pyridoxal or pyridoxamine. This does not necessarily mean that the ciliate does not use the phosphorylated compound, but only that some reaction or reactions appear to be limited to the nonphosphorylated vitamin (48).

Unlike the mammal, *T. geleii* W lacks enzymes for the synthesis of niacin. Tryptophan does not replace nor spare the vitamin (49).

Folic acid was earlier found to be a requirement for *T. geleii* W (57), and the ciliate is capable of responding to the conjugated forms [triglutamate and heptaglutamate (39)]. Enzymes are lacking for the condensation of the pteridine portion of the molecule to the *p*-aminobenzoyl glutamic acid portion. Likewise the ciliate cannot perform the specific peptide linkage between the carboxyl group of pteroic acid and the glutamic amino (39).

Recently it has been found that the folic acid analogues, 4-amino-pteroylglutamic acid (PGA) and N^{10}-methylpteroylglutamic can replace folic acid although inefficiently (48). This may mean that *Tetrahymena* possesses enzymes capable of oxidatively deaminating position four and demethylating position ten (52). When, however, both substitutions occur simultaneously (4-amino-N^{10}-methyl PGA) inhibition results. Inhibition is also produced by 4-amino-9,10-dimethyl PGA and 4-amino-9-methyl PGA. In the latter case, the inhibition appears to be strictly competitive, as PGA causes release of inhibition in a regular fashion. On the other hand, while PGA releases the inhibition of the triply substituted analogue, the inhibition index does not remain constant. A constant inhibition index is obtained when 4-amino-9-methyl PGA inhibition is released by the citrovorum factor, but the latter is no more effective than PGA against 4-amino-N^{10}-methyl PGA.

Protogen (100) is the only chemically unknown organic component required by *Tetrahymena*. Protogen has been identified with the acetate factor and the pyruvate oxidation factor for lactic acid bacteria (94). It functions in the production of acetate in *Tetrahymena* also, but in addition is required for some other vital function or functions (50). Acetate will spare, but not replace protogen. Recently it has been found that the ciliates rapidly die unless a certain amount of protogen is present in the medium. The inclusion of acetate, while it does not itself allow optimum multiplication with limiting protogen, does prevent death. Other acids associated with Krebs cycle are implicated (48a).

Unlike mammals and birds, *Tetrahymena* has lost enzymes for the synthesis of certain purines and pyrimidines (42, 46, 47, 54). The guanine molecule is an absolute requirement for all strains of *T. geleii* so far studied. In *T. geleii* W, adenine and hypoxanthine, and their nucleosides and nucleotides, spare but do not replace guanine. Xanthine and uric acid are inert. *T. geleii* W does not possess xanthine oxidase as judged by the following

observations (48a): The oxidation of hypoxanthine to xanthine is a reversible reaction via xanthine oxidase, and one would therefore expect some sparing action from xanthine. Hypoxanthine appears to be aminated to adenine and never seems to be oxidized to xanthine, for it is equal to adenine, on a molar basis, in sparing activity. Finally, it has been shown that 2-amino purine is oxidized in position 6 by xanthine oxidase (63). If the enzyme were present in *Tetrahymena*, then 2-amino purine should replace guanine in the diet. This is not the case, however, as 2-amino purine is quite inert.

Many other purine analogues have been studied in attempts to understand the enzymes of the purine system of *Tetrahymena* (Table 1). The

TABLE 1

ACTIVITY OF SUBSTITUTED PURINES OR PURINE ANALOGUES ON *Tetrahymena geleii* W (47, 48, 54)

Replacement*	Sparing†	Inhibition‡	Inert
1-methyl-guanine	adenine	8-azaguanine	xanthine
	hypoxanthine	8-azadiamino-purine	uric acid
1-methyl-xanthine	7-methyl-guanine	8-azadenine	8-azahypoxanthine
		theobromine	2-hydroxy-azapurine
2,6-diamino-purine	1,7-dimethyl-guanine	theophylline	7-methylxanthine
		paraxanthine	3-methylxanthine
8-methyl-guanine		caffeine	2-thio-6-ketopurine
		benzimidazole	8-ethylguanine
		o-phenylenedi-amine	8-methyladenine
		5-mercapto-azadenine	4,6-diaminobenzimidazole
			2-aminopurine (48a)

* Replaces guanine with from 75 per cent activity (1-methylguanine) to 4 per cent activity (8-methylguanine).

† Growth increases in the presence of suboptimal guanine.

‡ Methylxanthines not released by any purine.

triazolo analogues have proven most interesting (47, 54). These are analogues of purines wherein the 8 carbon of the imidazole portion of the ring is replaced by a nitrogen. It was found that the triazolo analogue of hypoxanthine (hypoxanthazolo or 8-azahypoxanthine) was inert; that of 2,6-diaminopurine inhibitory (inhibition index of about 27); that of adenine (8-azadenine) more inhibitory (index of about 7), and of guanine (guanazolo or 8-azaguanine) extremely inhibitory (index of 0.02). These inhibitions were shown to be competitive and the index of less than one for 8-azaguanine was accounted for on the basis of incorporation into the nucleoproteins of the cell (47). Further evidence was obtained tending to substantiate this theory, when it was found that the reduction of metabolic activities by

lowering of any of a number of essential metabolites (either by restriction or by specific antimetabolites) reduced markedly the inhibitory action of 8-azaguanine (53). Inhibition appears to depend upon incorporation. These experiments on *Tetrahymena* have been used as models for trials on neoplasms in mice (55), and it appears that certain mouse tumor cells differ qualitatively from normal cells in their purine metabolism (56).

In the pyrimidine system, certain enzymes present in higher animals appear to be missing in *Tetrahymena*. Of the normally occurring pyrimidine bases, only uracil will satisfy its pyrimidine requirement. Cytosine is inert, while thymine cannot be demethylated (46). Evidence is presented for the biosynthesis of thymine from nonpyrimidine precursors. Pteroylglutamic acid is active in this reaction (54). While cytosine is inert, cytosine nucleoside or nucleotide is sufficient to satisfy the pyrimidine requirement of *Tetrahymena* (46). Oxidative deamination of position 6 can be carried out only if the riboside linkage (position 3) is present. On the other hand, *Tetrahymena* does not possess decarboxylating enzymes for position 4 as evidenced by the fact that orotic acid (4-carboxyuracil) is inert (54).

Many pyrimidine analogues have been investigated for their activity on *Tetrahymena* and these are given in Table 2.

A number of studies have appeared dealing with the metabolism and respiration of various strains of *T. geleii*. Thus, Chaix *et al.* (9) have studied the effects of various organic substrates on *T. geleii* L. They found that oxygen uptake was increased about 50 per cent by butyric and acetic acids. Glucose, ethanol, propionic acid, isobutyric acid, caprylic acid, palmitic acid, homocysteine, and methionine produced moderate increases in oxygen uptake, while formate, succinate, stearate, citrate, α-amino butyrate, acetoacetate, α-hydroxybutyrate, and crotonic acid had little effect. Butyl alcohol, caproic acid, and malic acid had a slight depressing effect and protocatechol was very inhibitory. Linoleic acid caused inhibition and lysis. Seaman (92) has reported that malonate inhibits oxygen uptake in *T. geleii* S, and that this inhibition is released by fumarate, succinate, and α-ketoglutarate. Pyruvate increases oxygen uptake.

When *T. geleii* S (*Colpidium campylum*) was grown in fat-free peptone, no cholesterol was found (91). Fatty acid and lipid phosphorus remained constant over a seven-day period. Cytochrome-*c*, diphosphopyridine nucleotide, glutathione, and adenosinetriphosphatase were quantitatively determined in *T. geleii* S protoplasm grown in peptone (90). More recently (93), evidence from fluoride, malonate, and arsenite inhibition studies on *T. geleii* S has been presented, which indicates that this ciliate synthesizes both lipids and carbohydrates from acetate by independent routes. Acetaldehyde is thought to be the "active" two carbon compound in lipid formation, rather than acetylphosphate. This may explain, in part, the failure of *T. geleii* W to utilize coenzyme A (48) which has been shown to mediate phosphorylation of acetate and its further metabolism.

TABLE 2

ACTIVITY OF SUBSTITUTED PYRIMIDINES ON *Tetrahymena geleii* W (46, 48, 54)

Replacement*	Sparing†	Inhibition‡	Inert
1-methyl-uracil	4-methyl-uracil	2-thio-4-aminopyrimidine	cytosine
3-methyl-uracil		5-hydroxyuracil	thymine
5-bromo-uracil (?)		2-thio-6-ketopyrimidine	orotic acid
5-nitro-uracil (?)		2,4-diaminopyrimidine	isocytosine
		5-aminouracil	barbituric acid
		2,4-diamino-6-methyl-pyrimidine	6-methyl-thiouracil
		2,4-diamino-5-formyl-amino-6-hydroxy-pyrimidine	5-amino-barbituric acid
		2-aminopyrimidine	5-methylcytosine
		5,5-diethylbarbituric acid	1,3-dimethyluracil
		5-ethyl-5-phenyl-barbituric acid	4-hydroxypyrimidine
		5-ethyl-5-isopropyl barbituric acid	4,5-dihydroxy-pyrimidine
		5-ethyl-5-N-butyl-barbituric acid	5-methyl-6-hydroxy-pyrimidine
		5-ethyl-5-(1-methyl butyl) barbituric acid	2,4,6-triamino-5-formylamino-pyrimidine
		5-ethyl-5-cyclohexenyl barbituric acid	2,4-diamino-5-acetyl-amino-6-hydroxy-pyrimidine
		3,5-dimethyl-5-cyclo-hexenyl barbituric acid	2,4-diamino-5-propionylamino-6-hydroxypyrimidine
		6-N-propylthiouracil	
		5-methyluracil	
		bis-desazouracil	

* Replaces uracil, but with 1 per cent activity or less.

† Growth increases in the presence of suboptimal uracil.

‡ Inhibition of first eight compounds released by uracil, others not released by any pyrimidine.

A report has appeared on the inhibitory action of ω-methyl-pantothenic acid on *T. geleii* H and its release by pantothenic acid (88). High concentrations were required to produce inhibition and no index could be calculated due to the indeterminate amount of pantothenic acid present in a crude fraction of the medium (cerophyl extract).

Chaix *et al.* (10) tested the action of the four isomers of hexachlorocyclohexane on *T. geleii* L. All isomers caused lysis, although the Δ-isomer was most potent. This is in contrast to the growth inhibitory action on inositol-

less *Neurospora* (19) where the γ-isomer was most effective. It was found that the addition of inositol had no protective effect. Lysis was prevented by Tween 20 or Tween 80. Chaix *et al.* suggest that these surface-active agents coat the cell, thereby preventing the entrance of the hexachlorocyclohexane molecules. It may be, of course, that the hexachlorocyclohexane molecules are themselves "coated" or bound in some way by the Tweens.

T. geleii H has been proposed as an assay organism for certain amino acids (85, 86) and as a means of determining the "biological value" of proteins (87). It appears that such applications are premature since the basal medium used was not optimal for growth (13). Moreover, the use of acid production as a means of growth determination of these ciliates is unsound, because a low pH causes death and lysis.

Table 3 presents a summary of certain biochemical information obtained with *Tetrahymena*.

TABLE 3

SYNTHETIC ABILITIES OF *Tetrahymena geleii* FOR SPECIFIC ORGANIC COMPOUNDS

Unable to Synthesize* (dietary requirement)	Synthesize† Slowly (stimulatory)	Synthesis Demonstrated (by identification or by sparing action)
histidine (38a, 40, 43, 86, 16)	serine (40)	cysteine and cystine (41, 22)
isoleucine (38a, 40, 43, 16)	arginine (40)	homocysteine (41, 22)
leucine (38a, 40, 43, 16)	valine (40)	cystathionine (48a)
lysine (38a, 40, 43, 16)	thiamine (48)	ornithine (48)
methionine (38a, 40, 43, 16, 22)		citrulline (48)
phenylalanine (38a, 40, 43, 16)		tyrosine (38, 48)
threonine (38a, 40, 43, 16)		biotin (45, 48)
tryptophan (38a, 40, 43, 86, 16)		choline (45, 48)
pantothene (45, 16)		inositol (48)
niacin or niacinamide (45, 16, 49)		vitamin B_{12} (48)
pyridoxal or pyridoxamine (44)		thymine (54)
riboflavin (45)		adenine (47, 54)
pteroylglutamic acid (57, 39, 45)		adenosinetriphosphatase (90)
protogen (43, 100, 50)		fatty acids (93)
guanine (47, 54)		cytochrome-*c* (90)
uracil or cytidine (46, 54)		diphosphopyridine nucleotide (90)
		glutathione (90)
		acetate (93)
		carbohydrates (93)
		succinate (106, 108)
		lactate (106)

* Complexes containing these moieties are not listed.

† Under special circumstances.

Conclusion

This review will, it is hoped, point out the excellent opportunities which exist for further work, using protozoans as experimental material. Even with the few types so far studied under controlled conditions, it has been found that many enzymatic differences between types exist. When more of these differences have been exploited from the chemical and metabolic standpoints, a clearer idea of general nutrition and metabolism will emerge.

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PHYSIOLOGY OF THE ALGAE[1]

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The algae have been used as experimental tools in the investigation of a number of physiological processes. *Chlorella* and *Scenedesmus* have become standard organisms for theoretical work on photosynthesis. Important studies on permeability include the work of Collander & Barlund (34) on penetration of nonelectrolytes in *Chara* and the work of Osterhout [e.g. (143)] on the penetration of weak electrolytes in *Valonia*. The phenomenon of oxidative assimilation was first demonstrated by Barker (12) on the colorless alga, *Prototheca*. The work of Hutner *et al.* (94) has led to the use of *Euglena* as an assay organism for vitamin B_{12}. In view of such widespread use of selected forms, it is indeed surprising that this review should mark the first attempt to consolidate knowledge of the physiology of the algae as a group. Certain aspects of the field are covered by a review on algal pigments by Cook (32), a discussion of the phytoflagellates by Hutner & Provasoli (92) and treatments on algal culture by Bold (17), Pringsheim (170), and a symposium volume (24).

As commonly defined, the algae include a "heterogeneous assemblage of simple plants" (183). In addition to the pigmented autotrophic forms classically included in the group, it is desirable to include in the present discussion pertinent data on the colorless counterparts of a number of the green, autotrophic species. Physiology has diffuse limits which are not defined by this review; rather, it is the intent to build a core of information based upon nutrition, metabolism, and growth of the algae.

Methodology

Most of the work on algal physiology has utilized growth as a measure of overall nutrition or metabolism. In few cases has proper attention been paid to the requirements for critical growth assay as summarized by Monod (122). In addition to the conventional problems of microbiology, there are two technical problems which have not always been adequately solved: provision of light and of carbon dioxide. Fortunately, rather low levels of illumination (100 to 300 foot candles) are adequate to provide for light-saturated growth in *Chlorella* (124) and probably for other algae as well. However, due to mutual shading of the cells, the effective light intensity may be considerably less than the incident intensity except in very thin suspensions. "Light from a north-window" is seldom adequate for critical

[1] The survey of literature pertaining to this review was concluded in March, 1951.

[2] With the assistance of Anita Mol McCoy. Aid in preparation was provided by the Atomic Energy Commission under Contract AT-(40-1)-1039.

work. Still more serious has been the lack of attention paid to provision of carbon dioxide. Cultures growing photosynthetically in cotton-plugged flasks are growth-limited by diffusion of carbon dioxide. They may afford qualitative information on utilization of organic substrates but are entirely useless in providing interpretable quantitative data. Aeration with air may

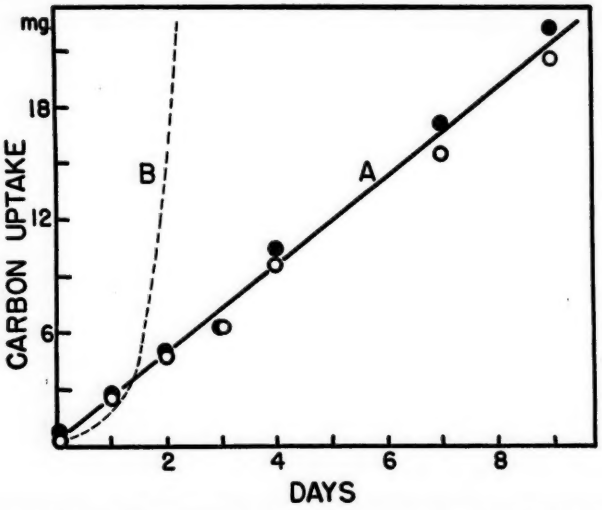

FIG. 1.—A. Uptake of carbon as carbon dioxide from air by 50 ml. of 0.2 *N* NaOH in cotton-plugged 250 ml. Erlenmeyer flasks under continuous shaking. The slope of the curve is the rate of carbon uptake when the carbon dioxide concentration in the fluid is zero.

B. Experimentally observable growth curve for *Chlorella* when adequate carbon dioxide provision is maintained by aeration with 5 per cent carbon dioxide.

In a cotton-plugged flask the growth curve would always be limited to the region below Curve A. The data are presented to show that diffusion from air through cotton plugs does not provide adequate carbon dioxide provision for quantitative work.

be adequate at low algal populations, since it has been shown that *Chlorella* and *Hormidium* are photosynthesis-saturated at about 0.03 per cent carbon dioxide (45, 90) and that *Scenedesmus* is growth-saturated at this level (147). However, if the rate of carbon dioxide-uptake is high it becomes physically impossible to maintain the effective carbon dioxide concentration in the medium even close to 0.03 per cent (cf. 128, 129). A safer procedure, widely used in culturing algae for photosynthesis experiments, is to aerate

with 1 to 5 per cent carbon dioxide in air. Such gas mixtures may be purchased in cylinders or prepared by continuous flow devices (132, 147, 179). An alternative procedure introduced by Osterlind (147) utilizes sealed test tubes provided with high bicarbonate concentrations. Other experimental problems of algal culture have been treated in the recent symposium volume (24). A continuous-culture technique has been devised (132); the chemostat invented by Novick & Szilard (139) should find wide use in deficiency studies.

Inorganic Nutrition

Major salts.—Following the conventions of plant physiology, it is useful to treat inorganic requirements in terms of the major salt constituents and trace elements; nitrogen nutrition is left for a separate section. Systematic studies of composition of the media have been made by Chu (28) for a number of plankton forms, by Gerloff *et al.* (73, 74) for several blue-greens, by Ondratschek (142) for a number of flagellates, by Pratt (159) for *Chlorella vulgaris*, by Rodhe (177) for *Ankistrodesmus*, and by Osterlind (147) for *Scenedesmus*.

Commonly used algal media (cf. 16) typically provide potassium, magnesium, sulfate, and phosphate (in addition to a nitrogen source). Pratt (163) has observed a sodium:potassium balance effect on photosynthesis of *Chlorella* in bicarbonate buffers. Emerson & Lewis (47) have reported a high sodium, low potassium requirement for *Chroococcus*, but this does not appear to be a general phenomenon.

Growth and metabolic effects of potassium have been studied intensively by Pirson (155, 156, 157) in *Chlorella* and *Ankistrodesmus*. Potassium-deficient cells showed lower rates of growth and photosynthesis and higher rates of respiration. For growth, potassium was partially replaceable by rubidium but not by lithium, sodium, or cesium. Rapid restoration toward normal photosynthetic and respiratory rates of potassium-deficient cells could be observed following potassium or rubidium addition in manometric experiments. From Pirson's work it appears that correlated studies of various cellular processes in *Chlorella* or other algae might prove valuable in elucidating the general and unique role of potassium in biological systems.

Scott (179, 180, 181, 182) studied mineral composition and cation exchange in *Chlorella*. Most of the cellular potassium and magnesium is bound and not replaceable; in contrast, sodium and calcium are almost completely replaceable. Hutner (91) has introduced a "chelate extinction technique" applied to *Nitzschia* for determination of minimal calcium or magnesium level. The total magnesium concentration requirement was determined at various levels of citrate; the data allow extrapolation to zero citrate concentration where total magnesium equals the ionic magnesium. Evidence has been presented by Mandels (115) that sulfur-deficiency specifically affects chlorophyll formation in *Chlorella*.

Because it is frequently an ecologically limiting factor (cf. 29), the phos-

phate requirement has received considerable study. Data on *Euglena* and on several of the Chlorophyceae indicate a high phosphorus content of about 2 per cent (1, 102). Ketchum (99) has studied the interplay of phosphate and nitrate concentrations on the ratio of phosphate:nitrate uptake from sea water by *Nitzschia*. Ketchum (100) and Scott (182) studied phosphorus-deficiency in *Chlorella* and *Nitzschia*. In phosphate-free media, the algae continued to grow until the cells had acquired a phosphorus debt for more than half of their usual content; addition of phosphate resulted in rapid uptake, restoring the normal cellular level. Extreme variation in the phosphorus requirements of different plankton forms has been noted by Rodhe (177). Addition of phosphate at a level of 5 μg. per liter to water of a fresh-water lake inhibited growth of two Chrysophyceae, *Dinobryon* and *Uroglena;* addition of 2 to 20 μg. per liter was necessary to obtain maximum growth of *Asterionella;* 500 to 1,000 μg. per liter addition was needed to obtain maximum growth in *Scenedesmus*.

Diversity of natural habitat leads to expectation of a wide range of adaptation to total salt concentration. For the plankton form *Pediastrum*, Chu (28) noted an optimum total salt concentration of about 100 p.p.m. In contrast, Pratt & Fong (165) observed an optimum at 10,000 p.p.m. for *Chlorella* and the Pringsheims (171) have reported an optimum of about 25,000 p.p.m. for *Porphyridium cruentum*. The low salt tolerance of the plankton forms imposes severe restrictions on physiological work; continuous-dilution methods would appear desirable. It is not surprising that the more tolerant and hardy species have been selected more frequently for detailed study.

Trace elements.—The soil-water culture technique and use of soil extract championed by Pringsheim (e.g., 169, 170) has proved extremely useful in exploratory studies. The role of the soil constituents may be attributed to provision of possible organic growth factors and, more generally, to the provision and restriction of ionic concentrations of trace elements by chelation and adsorption exchange. Refinement of medium composition leads to difficulties in maintaining necessary trace elements in the ionic concentrations between the narrow limits of requirement and toxicity and in preventing formation of difficultly reversible precipitates or colloidal complexes. Hutner and co-workers (e.g., 93) have extended and refined chelation techniques on the principle that the easily reversible chelate complex provides a buffer system for the heavy metals, maintaining their ionic concentrations within desirable limits. The exacting requirements for trace element studies as laid down by Hutner *et al.* (93), seldom have been met.

The universal requirement for iron needs no documentation. Effects of iron-deficiency have been studied in relation to nitrogen metabolism in *Chlorella* by Noack & Pirson (137) and by Alberts-Dietert (2); iron apparently plays a role subsidiary to that of manganese. Metabolic effects, other than chlorosis, should be explored. One might note, for instance, the observation of Whelton & Doudoroff (200) that an abnormal accumulation of

pyruvic acid may occur in iron-deficient cultures of *Pseudomonas saccharophila*.

In a study of ecological significance, Rodhe (177) has shown that natural lake waters analyzing 0.1 to 0.7 p.p.m. total iron contained only 0.01 to 0.03 p.p.m. iron responding to phenanthroline; subsequent bioassay, using iron-starved *Scenedesmus*, gave results agreeing with the phenanthroline analysis. The author has repeatedly demonstrated that a freshly prepared Knops solution with 0.1 p.p.m. iron supports adequate growth of *Chlorella;* after several months the same stock solution, although still optically clear, gives cultures with deficiency symptoms correctable by additional iron. Apparently iron readily forms colloidal complexes which are only sluggishly reversible and biologically unavailable.

Manganese also appears to be a universal requirement of the algae. It may be a limiting ecological factor (85). Manganese-deficiency has specific effects on nitrogen metabolism in *Chlorella*, and particularly on nitrate assimilation (2, 137). Pirson (157) has demonstrated a three-fold increase in rate of photosynthesis within 2 hr. after addition of manganese to deficient cells of *Chlorella*.

Zinc has been demonstrated as a growth requirement for a number of flagellates (142) and for *Chlorella* (187). It has been demonstrated frequently that the algae do not require calcium at the high level demanded by higher plants. Evidence for requirement of calcium as a trace element has been presented by Ondratschek (142) for several flagellates, by Noack *et al.* (138) for *Chlorella*, and by Waris (197, 198) for *Micrasterias* and *Eremosphaera*. By use of their chelate-extinction technique, Hutner & Provasoli (92) have established a 5 p.p.m. calcium requirement for *Chlorella* and *Euglena*. A molybdenum requirement has been demonstrated for a number of nitrogen-fixing blue-greens (18, 19) but not generally among the algae, although it is often incorporated in nutrient media. Copper and boron are also included in many nutrient media, although no definite requirement, except for one possible case with copper (82), has yet been shown. Copper has been shown to be toxic for photosynthesis in *Chlorella* at the very low concentration of $10^{-7}M$ (78). At the same time, it may be anticipated as a trace element: a series of poisons of copper-catalysts have been shown to inhibit photosynthesis in Chlorella (77); the copper in the leaves of higher plants is concentrated in the chloroplasts (136); and the copper enzyme polyphenol oxidase is localized in chloroplasts (9, 10).

Nitrogen Nutrition and Metabolism

The algae have a nitrogen content comparable to that of other microorganisms and considerably higher than that of most higher plants. Nitrogen analyses reported for *Anabaena* (55), *Nitzschia* (102), *Chlorella* (37, 102, 134, 185), *Scenedesmus* (102), *Stichococcus* (102), and *Euglena* (36) run from about 7 to 11 per cent of the dry weight for freely growing cells.

Free nitrogen.—Nitrogen-fixation has been demonstrated with reason-

able certainty in four genera of the blue-greens, *Anabaena*, *Aulosira*, *Cylindrospermum*, and *Nostoc*, all members of the Nostocaceae [cf. Fogg (53)]. It is anticipated in other blue-greens from ecological considerations; it has been sought and not found in *Phormidium* (41) and may be a characteristic not common to all members of the group. A number of blue-greens have developed symbiotic relationships with fungi and higher plants. Winter (205) has reported that strains of *Nostoc* isolated from *Gunnera* and from a cycad require carbohydrate for growth and nitrogen-fixation and give rise to soluble nitrogen in the culture medium. Occurrence in the culture filtrate of a considerable portion of the fixed nitrogen, noted also in free-living forms (41, 54), is apparently a true excretion since it is greatest in young cultures (53). Nitrogen-fixation is suppressed by an available nitrogen source in the medium (6, 41); it may be accomplished in the dark when a utilizable carbon source is available (6, 205). Comparative data on growth and metabolism with and without fixed nitrogen are not yet available. Relationships between hydrogenase activity and nitrogen-fixation observed in the photosynthetic bacteria (e.g., 96, 97) should be examined also in the blue-greens. The long debated question of nitrogen-fixation in the Chlorophyceae is under investigation in those forms known to have a hydrogenase system. To date the results have been negative (59).

Nitrate and ammonium ion.—A number of observations on various members of the Chlorophyceae support the generalizations (*a*) that both nitrate and ammonium ion support growth in light or in the dark when a utilizable carbon source is available and (*b*) that in the presence of both ions, ammonium is preferentially absorbed (37, 109, 114, 150, 151, 166). Nitrogen-starved *Chlorella* may take up nitrate and ammonium simultaneously (193); Zobell (208) has observed a simultaneous uptake of ammonium and nitrate by the diatom *Nitzschia*. Uptake of ammonium is typically accompanied by decreasing pH, probably because the penetrating species is actually NH_4OH; conversely, an increasing pH attends nitrate absorption presumably due to exchange of nitrate and hydroxyl ions or to simultaneous penetration of H^+ and NO_3^- as an ion pair. In practice, pH stabilization is more difficult with ammonium so that the choice of nitrate in culture media is more general.

Ammonium is utilized quite generally by autotrophic organisms. Special inhibiting effects of ammonium have been noted by Osterlind (147) for growth of *Scenedesmus* and by Greenfield (78) for photosynthesis of *Chlorella;* inhibition by high concentration of undissociated NH_4OH may be rather general. Ability to utilize nitrate is widely distributed among the various groups of the algae; exceptions occur in the colorless flagellates, in heterotrophic growth of several of the green flagellates (111, p. 91), and in *Euglena gracilis bacillaris* which will not grow autotrophically with nitrate as the sole nitrogen source (36).

Other nitrogen sources.—Systematic study of other possible nitrogen sources has been made by Reinhardt (174) for a number of heterotrophic

flagellates, by Ludwig (108) for *Chlorella*, and by Algéus (3) for a number of the Chlorophyceae. Algéus (4, 5) further investigated utilization of glycine and alanine by a number of green algae. He recognized two groups, a *Chlorella*-type in which deamination limits the rate of nitrogen assimilation and a *Scenedesmus*-type in which there is a more rapid deamination and excretion of NH_3 into the medium. Specific amino acid requirements may occur in certain forms (e.g., 154).

The algae, as the higher plants, exhibit a careful conservation of nitrogen. Pearsall & Billimoria (149) reported significant loss of nitrogen from cultures of *Chlorella* but this was not confirmed by subsequent work of Allison *et al.* (7). Warburg & Negelein (195) observed an ammonium excretion accompanying nitrate reduction in *Chlorella* but only under extreme conditions of high acidity and zero carbon dioxide. Under growth conditions *Chlorella* reduces nitrate only at a rate determined by the requirements of cellular synthesis (37). Myers & Johnston (134) have reported a 95 per cent recovery in *Chlorella* cells of the nitrogen removed from nitrate in the medium during growth.

Data on *Chlorella* allow one to outline a pattern of nitrogen metabolism probably applicable to other algal forms. When growing under constant conditions limited only by light intensity, *C. pyrenoidosa* produces cells of a constant composition of 10.8 per cent nitrogen or 5.7 C/N (37). If such cells are starved by aerobic incubation in darkness, they lose easily accessible carbohydrate reserves (38); on return to growth conditions, starved cells show nitrogen assimilation only after a considerable period of photosynthesis which presumably restores the original C/N balance (133). Conversely, cells suddenly exposed to light-saturating intensities for photosynthesis show an abnormally high C/N assimilation ratio; on return to the original growth-limiting light intensity such cells show an unusually high rate of nitrogen assimilation; a similar and even more pronounced effect occurs in nitrogen-starved cells (133). After removal or depletion of a nitrogen source, a *Chlorella* culture continues to increase in dry weight giving rise to cells with a low nitrogen content (100, 102, 185), a reduced capacity for photosynthesis (88), and an ability to assimilate nitrogen at an unusually high rate (100, 193). Similar but less extensive data on *Nitzschia* (100) and on *Ankistrodesmus* (157) support the generality of the phenomenon. Little information is available on the biochemistry of nitrogen metabolism in the algae. Keto acids, produced in regenerative fashion by a condensation cycle, generally provide the key components for carbohydrate, amino acid, and lipid synthesis. The principal business of this metabolic machinery is the production of cellular material of high nitrogen content. A fraction of the labeled carbon dioxide taken up in short periods of photosynthesis by *Chlorella* and *Scenedesmus* appears in amino acids and insoluble protein (15). Ammonia and carbon dioxide may well act as competitors for components of the condensation cycle (cf. 188); a nitrogen deficiency closes the metabolic channels

of amino acid synthesis, giving rise to extra carbohydrate and lipid synthesis as a shunt or overflow metabolism in the sense of Foster (56). This is only a restatement in terms of mechanism of a generalization made long ago by Beijerinck (15a).

Carbon Nutrition and Metabolism

Carbon dioxide.—Carbon dioxide is the principal natural carbon source of most algae. Arguments that the penetrating species is the undissociated form rather than HCO_3^- or $CO_3^=$ have been summarized by Rabinowitch (173, p. 196). Osterlind (144, 147) has obtained extensive data on growth of *Scenedesmus* as a function of pH under various concentrations of CO_2 in the aerating gas. The data must be interpreted either in terms of pH effects which depend also on the CO_2 concentration or in terms of participation of HCO_3^- as the penetrating species. In subsequent experiments with *Chlorella* (145) the results were quite different and were interpreted to indicate negligible participation of HCO_3^-. The latter conclusion also obtains from the data of Tseng & Sweeney (192) on the red alga *Gelidium.* Differences between *Scenedesmus* and *Chlorella* observed by Osterlind (146) could not be explained in terms of carbonic anhydrase activity and permeability differences were postulated.

In view of the carbon dioxide requirement of *Escherichia coli* established by Lwoff & Monod (113) possible effects of carbon dioxide on heterotrophic metabolism should be sought in the algae. Work of this laboratory (36, 153) indicates that dark metabolism of *Chlorella* or *Euglena* is not generally inhibited by zero carbon dioxide but that there are a number of interesting effects observed in zero carbon dioxide plus utilizable organic substrate plus light. It has been noted also that, as previously reported (e.g., 1), *Euglena gracilis* cannot utilize glucose for growth under the usual conditions of culture; however, in an atmosphere of 5 per cent carbon dioxide and with ammonium as the nitrogen source it attains excellent growth after a lag period. Effects of carbon dioxide were observed by Iggena (95) and by Luksch (109), but the experimental methods were not exacting. An effective zero carbon dioxide concentration for growth experiments can be maintained only by vigorous aeration with alkali-scrubbed air; control cultures should be provided 1 to 5 per cent carbon dioxide by aeration. Effects of carbon dioxide on algal metabolism merit further exploration.

Organic carbon sources.—Extensive work on the utilization of carbon sources by the phytoflagellates has been reviewed by Doyle (43) and given more recent critical treatment by Hutner & Provasoli (92). Growth in light and in darkness on various carbon sources has been explored by Algéus (3) for a number of the Chlorophyceae, by Barker (11) for *Prototheca,* by Bristol-Roach (20, 21) for several soil algae, by Maher (114) for *Protosiphon.* Respiratory response to various substrates has been reported by Genevois (70, 71, 72) for a number of algae and by Myers (125) for *Chlorella.* The only possible

simple generalization is that the monosaccharides and acetate are the most widely utilized substrates. Because of the toxicity of free acetic acid, the utilization of acetate may easily be missed. A concentration of 0.002 *M* undissociated acetic acid completely inhibits photosynthesis in *Chlorella* and a medium containing 1 per cent sodium acetate markedly inhibits growth even at pH 6.8 (131).

Conservation of carbon and a minimal level of excretion is probably generally characteristic of the green algae. From carbon balance data on *Scenedesmus* (104) and on *Chlorella* (134, 185), it can be calculated that 95 to 98 per cent of the total carbon of cultures was recovered in the algal cells. Soluble photosynthate was sought but not found by Barker (13) in cultures of *Nitzschia*. It is inferred that the uptake of substrates which are also cellular constituents must be accomplished by one-way permeability or active transport. Emphasis is commonly placed on the role of autotrophs in raising the energy level of carbon; they also provide the mechanism of carbon accumulation and can ill afford wasteful luxuries of excretion.

Autotrophic metabolism.—Recent developments in photosynthesis disclose the biochemical pattern of the process (cf. 15). Uptake and reduction of carbon dioxide appear to be accomplished by a carboxylation and condensation cycle driven by the photochemical oxidation of water. Components of a condensation cycle, which provide the starting points for further syntheses, are thus indicted as the long sought "first products of photosynthesis." The classical specification of carbohydrate as the first product of photosynthesis has become an approximation of only historical usefulness.

Photochemical oxidation of water has submitted to study as the Hill reaction in which chloroplast preparations from higher plants or from algae photochemically reduce substituent oxidants such as Fe^{+++} ion. Reduction of components of the medium in light by intact cells of *Chlorella* in the absence of carbon dioxide has been demonstrated by Fan *et al.* (51), Holt & French (89), and Warburg & Lüttgens (196). A coupling of the Hill reaction and enzymatic carbon dioxide fixation has been accomplished by Vishniac (194) in a sub-cellular preparation.

Maximum rates of photosynthesis reported by Emerson & Green (45) for *Chlorella*, by Barker (13) and by Wassink & Kersten (199) for several diatoms, by Barker (14) for the dinoflagellate *Peridinium*, by Cramer (36) for *Euglena*, all fall within a range of about 10 to 50 volumes O_2 per hour per unit volume of cells. There is considerable variation, however, among different species of *Chlorella* (203) and within any one species depending upon previous culture conditions (88, 124, 129, 178, 204). The gas exchange observed at a maximum rate of photosynthesis is typically 10 to 20 times that due to endogenous respiration in the dark. Carbon dioxide saturation of photosynthesis in *Chlorella* (45), *Hormidium* (90), and *Nitzschia* (13) is attained at concentrations close to the 0.03 per cent provided by air. Light saturation of photosynthesis in *Chlorella* is attained in the range of 300 to

500 foot candles (e.g., 124, 203) but this characteristic will probably show wide variation depending upon light quality and the nature and concentration of the algal pigments.

The assimilatory quotient of gas exchange (CO_2/O_2), almost universally measured under conditions of light saturation, has yielded values of 0.9 to 1.0 in the algae (e.g., 13, 60, 124, 178, 192). This may be used as evidence for a net production of assimilates close to carbohydrate in composition. The author has stated (126) and vigorously contends that such values characterize only the metabolism occurring under the high light intensities chosen for reasons of experimental convenience. Invariably the algae have been cultured at low light intensities. When shifted to light saturation in a short-time manometric experiment their metabolism is forced into the channel of carbohydrate production. Where there are no organic excretory products one may predict with certainty that the steady-state quotient of gas exchange accompanying photosynthetic growth must coincide with the value that can be calculated for complete oxidation of the cells produced.

The work of Gaffron (63, 65) has shown that a number of algae have a latent hydrogenase system. By anaerobic incubation in the dark they may be adapted to use molecular hydrogen instead of water as the ultimate hydrogen donor for reduction of carbon dioxide at low light intensities. Increase in light intensity or in the oxygen tension causes reversion to normal photosynthesis. This phenomenon of photoreduction was originally described in *Scenedesmus*, *Raphidium*, and *Ankistrodesmus* but has subsequently been observed by Frenkel (58) in *Chlamydomonas Moewusii* and by Frenkel *et al.* (60, 61) in the blue-greens *Synechococcus* and *Chroococcus* and in the red alga *Porphyridium*. The nature of the assimilate arising from photoreduction has not yet been identified (cf. 23); there is not yet any evidence that this type of metabolism can sustain growth although it has been observed that *Porphyridium* will increase in dry weight and pigmentation (61). The hydrogenase enzyme and photoreduction appear not to be completely general in occurrence since they have been sought and not found in other closely related algae.

Heterotrophic metabolism.—When cells from a growing culture of *Chlorella* are incubated aerobically and without added substrate in the dark, their rate of respiration gradually decreases to a final value which may be maintained fairly constant for several days (38, 57). Attainment of the final rate corresponds in time to the disappearance of starch (38). Such starved cells will photosynthesize and respire glucose at normal rates but will not assimilate nitrogen nor proliferate. Only after a period of carbon assimilation do they again exhibit the metabolism of growing cells. An approximation to aerobic starvation is achieved by incubation at low light intensities so that the cells store only very limited quantities of easily utilizable endogenous substrate (cf. 71); similar procedure has frequently been used in preparation of cells for measurement of the quantum yield of photosynthesis. Because

of their tendency to store reserve materials, it is likely that algal cell preparations metabolically analogous to resting cells of bacteria are attainable only by starvation.

An oxidative assimilation of carbon sources into cellular materials has been demonstrated by the conventional manometric procedures in *Prototheca* by Barker (12), in *Chlorella* by Myers (125), and in *Scenedesmus* by Taylor (191). Starved cells of *Chlorella* and *Scenedesmus* may assimilate as much as 83 per cent of the glucose carbon; of the carbon assimilated by *Scenedesmus* about 80 per cent was recovered as polysaccharide hydrolyzable by acid but not by diastase. During growth, a much lower assimilatory efficiency of 49 per cent was observed in *Chlorella* (134). The summation of algal cell constituents is typically more reduced than carbohydrate (cf. 102, 185). It is to be expected that assimilation in growing cells will require oxidation of a greater fraction of the substrate as compared to the assimilation to carbohydrate accomplished in starved cells. It is also to be expected that anomalous values of the R.Q. of exogenous respiration will be observed (cf. 66), particularly if nitrate is being reduced (37).

Aerobic respiration in *Chlorella* continues at a normal rate down to the low partial pressure of about 5 mm. mercury (190). Under anaerobic conditions, an acid fermentation can be demonstrated in a number of algae (11, 64, 66, 69, 72, 120). In *Prototheca*, large quantities of lactic acid and some succinic acid are produced from exogenous glucose (11). Lactic acid is also produced in small quantities by *Chlorella* (120). Other features of anaerobic metabolism have been explored, particularly by the work of Gaffron. Prolonged anaerobiosis in the dark leads to an initial inhibition or anomalous gas exchange in the induction period of photosynthesis (62, 64, 120) which has been attributed to accumulation of fermentation acids or other reduced materials. In *Scenedesmus*, lactic and other acids and hydrogen are produced in endogenous fermentation; with added glucose there is increased production of hydrogen and lactic acid; and upon illumination the rate of hydrogen production is still further increased (69). When adapted anaerobically in the dark to a hydrogen metabolism, *Scenedesmus*, *Raphidium* (and presumably other algae having a hydrogenase system) will simultaneously reduce oxygen to water and carbon dioxide to carbohydrate if proper concentrations of H_2, O_2, and CO_2 are provided (67). Growth of a green alga under any anaerobic condition has not yet been demonstrated.

No unusual features of intermediary metabolism in the algae are indicated by the fragmentary data available. The expected phosphorylation cycle is evidenced by the distribution of cellular phosphorus constituents observed in *Euglena* by Albaum *et al.* (1) and in *Chlorella* by Emerson *et al.* (48); identification of phosphoglycerate as an early intermediate in photosynthesis by Calvin *et al.* (26), and confirmed by Fager *et al.* (50), establishes operation of the cycle also under autotrophic conditions. In the dark metabolism of *Chlorella* and *Scenedesmus* small amounts of C^{14}-labeled carbon dioxide taken

up have been traced into carboxyl groups of malic, succinic, fumaric, citric, glutamic, and aspartic acids (15, 26). The hypothesis of a condensation cycle which serves as the "protistan equivalent of the Krebs tricarboxylic acid cycle" (92) is well justified.

Relationships between heterotrophic and autotrophic metabolism.—The algae as a group are characterized by general ability to grow either autotrophically or heterotrophically. It is to be expected that all variations of degree of dependence on the two types of metabolism may occur in different species. *Euglena gracilis* cultured photosynthetically responds sluggishly to added substrates in the dark; when cultured in the dark it loses chlorophyll and on subsequent return to the light slowly regains photosynthetic ability (36). In several of the green flagellates, irreversible loss of chlorophyll may result from prolonged culture in the dark or treatment with streptomycin [Provasoli *et al.* (172); for a recent review see (92)]; there is no evidence of any significant alteration of growth or dark metabolism resulting from loss of photosynthetic ability. The numerous colorless counterparts of the green flagellates are interpreted as forms which have entirely abandoned an autotrophic metabolism. At the other extreme there is the recent report by Lewin (105) of obligate autotrophy in *Chlamydomonas Moewusii* which will utilize for growth no carbon source other than carbon dioxide.

Some of the algae, e.g., various species of *Chlorella* and *Scenedesmus*, have a very stable relationship between their heterotrophic and autotrophic metabolism. *Chlorella* grows well in the dark on added glucose, maintaining its pigment system intact (127); [note, however, a conflicting report for a different species (52)]; on illumination, a steady rate of photosynthesis is attained within a few minutes (88, 127). Cells cultured photosynthetically respond rapidly to added substrate in the dark. Bristol-Roach (22) made precise measurements of the growth rate of a species of *Scenedesmus* at 24.5°C. in the light, in the dark with added glucose, and in the light with added glucose. High and low light intensities were used. The data were expressed in terms of the specific growth rate constant in $\log_{10}$ units per day. At a low light intensity the rate was 0.15; in the dark plus glucose, 0.21; at low light intensity plus glucose, 0.38. Thus, the rate of growth supported by low light and glucose utilization, operating simultaneously, is experimentally the same as the sum of the growth rates observed independently, i.e., autotrophic and heterotrophic metabolism are additive in supporting growth. At high light intensity, the growth rate of 0.47 was not influenced by addition of glucose. Apparently a maximum growth rate, not limited by carbon assimilation, is attained under photosynthetic growth at light saturation. The latter phenomenon has been demonstrated also in *Chlorella pyrenoidosa* (Emerson's strain) (153). This species at 25°C. has a growth rate of 0.40 on glucose in the dark; at high light intensity the maximum growth rate of 0.85 is not affected by addition of glucose. Thus, under the latter conditions, the total rate of carbon assimilation from glucose and carbon dioxide cannot

proceed as the sum of the rates observed independently. Under steady-state conditions of growth at high light intensities, it should be possible to find a competition between glucose and carbon dioxide as carbon sources; experimental demonstration has not yet been accomplished.

There are many reports of acceleration of growth in light by added organic carbon sources. In no such case, however, is there clear evidence that the provision of carbon dioxide was adequate. It is a common observation, for instance, that if glucose is added to one of two illuminated cultures in cotton-plugged flasks more rapid growth will occur in the culture with glucose. The stimulation by glucose can be explained in terms of the added carbon assimilation provided by the oxidative channels of heterotrophic nutrition plus an increased rate of photosynthesis afforded by the extra carbon dioxide produced. Under conditions of light and carbon dioxide-saturation, however, no case has yet been found in which an organic carbon source accelerated growth. This is another way of saying that in the algae investigated to date, carbon assimilation may be better provided by photosynthesis than by oxidative assimilation.

The data of Iggena (95) have been used to support the assertion (66) that light stimulates growth on glucose in zero carbon dioxide, although the experimental technique was hardly adequate to maintain zero carbon dioxide in the cultures. Reinvestigation in this laboratory (153), using test tube cultures of *Chlorella* vigorously aerated with zero carbon dioxide, have failed to reveal any stimulation of light on rate of growth with glucose or acetate; on the contrary, light retards growth of *Chlorella* on acetate in zero carbon dioxide. In spite of such negative evidence on interrelationships between light and utilization of organic carbon, one significant possibility remains. It may be possible, and certainly would be desirable, to find an alga with the ability to use in photosynthesis an intact organic substrate in place of carbon dioxide.

Other methods of attack on metabolic problems.—Several of the standard biochemical techniques have found only limited application to the algae. Permeability restrictions prevent deductions about the course of metabolism from the nature of substrates which are or are not utilized. The use of homogenates or other subcellular preparations is almost unexplored. The technique of competitive inhibition has not found general application, although Wiedling (201) reported a *p*-aminobenzoic acid-reversible inhibition by sulfanilamide in a diatom. Study of metabolism by the analysis of genetic blocks in mutant strains is being applied to *Chlorella* by Davis (40) and by Granick (75, 76) and to *Chlamydomonas* by Lewin (106). Neither of these algae is completely suitable. Of very great usefulness would be the discovery of a green alga which can grow either autotrophically in the light or heterotrophically on various carbon sources in the dark and which reproduces through a sexual stage susceptible to genetic analysis.

Growth

In a relatively few investigations of growth of algae, the experimental technique has been adequate to allow a significant period of logarithmic growth and the data have been extensive enough to establish the logarithmic rate of growth. Some of these data have been collected and are tabulated

TABLE I

Comparative Growth Rates in Terms of Log_{10} Units per Day*

	Organism	k	temp. °C.	Conditions	Ref.
Bacteria	*Escherichia coli*	26.0	**	lactose broth	(116)
	Pseudomonas fluorescens	13.0	**	glucose broth	(116)
	Azotobacter chroococcum	6.0	**	urea, glucose	(116)
		1.3	**	sugar, mineral salts	(116)
Yeast	*Willia anomala*	6.0	30.0	glucose, yeast extract	(79)
Protozoa	*Tetrahymena geleii*	1.8	25.0	yeast autolysate	(152)
Algae	*Anabaena cylindrica*	0.32	23.0	light, CO_2, NO_3^-	(55)
	Chlorella pyrenoidosa	0.85	25.0	light, CO_2, NO_3^-	(124)
	(Emerson strain)	0.40	25.0	dark, glucose, NO_3^-	(153)
		0.21	25.0	dark, acetate, NO_3^-	(153)
	Chlorella vulgaris	0.49	23.0	light, glucose, NH_4NO_3	(150)
	(Wann strain)	0.29	23.0	dark, glucose, NH_4NO_3	(148)
	Scenedesmus quadricauda	0.88	25.0	light, CO_2, NO_3^-	(147)
	Scenedesmus costulus	0.47	24.5	light, CO_2, NO_3^-	(22)
		0.21	24.5	dark, glucose, NO_3^-	(22)
	Euglena gracilis	0.60	25.0	light, CO_2, NH_4^+	(36)
	(from Pringsheim)	0.25	25.0	dark, butyrate, NH_4^+	(36)
	Euglena gracilis	0.42	25.0	light, CO_2, NH_4^+	(36)
	var. *bacillaris*	0.42	25.0	dark, butyrate, NH_4^+	(36)
	Prorocentrum micans	0.20	18.0	light, seawater, soil extract	(14)

* A value of 0.30 corresponds to a generation time of one day.

** At the optimum temperature for each organism.

in Table I, together with similar data on other microorganisms. The algae are not rapidly growing organisms; and, in fact, those selected for experimental study probably are among the most rapidly growing members of the group.

Growth inhibitors.—The extensive work of Pratt (158, 160, 161, 162, 164, 165, 167) on *Chlorella vulgaris* (Trelease strain) has established the produc-

tion of an inhibitor of growth and photosynthesis by vigorously growing cultures. The inhibitor, called chlorellin, proved to be heat-unstable, diffusible through a collodion membrane, and extractable from dried cells by ethyl alcohol, ethyl ether, and petroleum ether; it has appreciable antibiotic activity against *Staphylococcus aureus*. The experimental method was based on cultures in Erlenmeyer flasks adequately aerated with 5 per cent carbon dioxide. Unfortunately, the use of thick suspensions probably led to a light limitation of growth which cannot be disentangled from inhibition due to the chlorellin. Winokur (202) observed greater maximum growth of the same organism under even less adequate illumination conditions. The phenomenon reported by Pratt is of interest and importance but there is as yet no evidence of its generality among the algae; on the contrary, the work of Winokur suggests that in other species of *Chlorella* the effect is absent or that it appears only at much higher densities of population. The precise nature of chlorellin has not been established. Antibacterials produced by *Chlorella pyrenoidosa* have been shown to result from photooxidation of unsaturated fatty acids (186).

Growth factors.—Growth factor requirements for the algae have been treated by Hutner & Provasoli (92 [see also Lwoff (110)]. Thiamine has been shown to be a requirement for the heterotroph *Prototheca* (8), for the obligate photoautotroph *Euglena pisciformis* (44), for *Euglena gracilis* growing heterotrophically in the dark (110, 112), and for *Euglena gracilis* var. *bacillaris* (94). By following the growth curves for the latter organism, it has been possible to demonstrate unequivocally an absolute requirement for thiamine both in the light with carbon dioxide as the carbon source and in the dark with any of a number of organic substrates (36). Ondratschek (140, 141) has reported thiamine requirements for heterotrophic growth (but not for autotrophic growth) of a number of flagellates; his results have been questioned by Lwoff (110) and ignored by Hutner & Provasoli (92).

B_{12} has been demonstrated as a requirement of *Euglena gracilis bacillaris* (194) and *Chlamydomonas chlamydogama* (154). With the former organism, Hutner *et al.* (94) developed a sensitive assay method which has been used by Robbins *et al.* (176) to determine B_{12} concentrations in soil, in pond water, and in other organisms. Attention is directed to the specificity, sensitivity, and simplicity of the *Euglena* B_{12} assay method.

Presumptive evidence has been cited for growth factor requirements in a blue-green and several other plankton algae (177), for several marine dinoflagellates (14), and for a diatom (84). The problem of whether or not indoleacetic acid (plant heteroauxin) has any significant effects on growth of the unicellular algae has already been treated by Algéus (3) more extensively than this muddled subject deserves and will not be considered here.

Relationships between growth and photosynthesis.—Attention should be called to an interesting relationship between photosynthesis and growth which can be deduced from data on *Chlorella* (124). Maximum rate of

growth is achieved at a light intensity of 100 foot candles; photosynthesis of cells grown at this intensity is light-saturated only above 400 foot candles. It appears that in progressing from 100 to 400 foot candles the cells can photosynthesize more rapidly but cannot grow any more rapidly. A similar conclusion is reached by comparison of rates of carbon assimilation calculated from the growth rate and from the maximum rate of photosynthesis. When reduced to an infinitely small time period, the maximum growth rate of 0.85 log units per day corresponds to an increase of 8.2 per cent per hr. One c.mm. of cells contains 0.24 mg. dry weight which is about 50 per cent carbon. Thus, as calculated from the maximum growth rate, 1 c.mm. of cells can assimilate 10 μg. of carbon per hr. The same cells (grown at 100 foot candles) can attain a maximum rate of photosynthesis of 38 c.mm. CO_2 per c.mm. per hour. This corresponds to an assimilation of 20 μg. of carbon per hour by one c.mm. of cells. Both types of reasoning lead to the anomalous conclusion that *Chlorella* can photosynthesize faster than it can grow. [Excretory products are negligible, even at high light intensity (134).] The following explanation is proposed. Cells grown at 400 foot candles are over twice as large as cells grown at 100 foot candles but without any greater chlorophyll content per cell (123). Photosynthesis is measured in short time manometric experiments in which the cells do not maintain a steady-state metabolism. They are restricted in rate of division but not in size and adjust to the more than adequate carbon assimilation by producing extra quantities of reserve materials. As specifically applied to *Chlorella*, the argument indicates that the maximum growth rate is not established by the rate of carbon assimilation; nor is it established by the rate of nitrogen assimilation (133); the maximum rate of cell division must be determined by internal regulating mechanisms as yet unknown. The argument also may be extended to the hypothesis that the algae, lacking mechanisms of excretion, have a highly versatile internal metabolism which adjusts readily to either luxurious or marginal nutritional economy, giving rise to wide variations in cell size and composition.

Algal Composition

The dry weight of unicellular green algae typically amounts to about 20 to 25 per cent of their packed volume obtained on centrifuging (cf. 102). The ash content typically falls in the range of 5 to 10 per cent of the dry weight. Spoehr & Milner (185) have devised an expression, the R-value, for the percentage degree of reduction of carbon running from zero for CO_2 to 100 for CH_4. From the R-value, calculated on the basis of carbon, hydrogen, and oxygen analysis, and from the nitrogen content, the proportion of protein, carbohydrate, and lipid can be estimated. Estimates for lipid content showed good agreement with actual lipid analysis (121). Control of culture conditions, particularly the provision of nitrogen, allowed production of *Chlorella* cells varying in R-value and per cent estimated constituents as follows (185):

R-value	*Protein* %	*Carbohydrate* %	*Lipid* %
38	58	37	5
63	9	6	85

A very much smaller range of variation was observed in a number of different green algae grown under uniform conditions (102).

Lipids.—Analytical data on the lipids from diatoms, *Chlorella*, and *Scenedesmus* indicate no unusual feature nor any marked species differences (30, 42, 98, 107, 121). The bulk of the lipid is saponifiable and with a high content of C_{16} and C_{18} acids, a large fraction of which is unsaturated. Sterols have been obtained from most of the algal groups except that they have been sought and not found in the blue-greens (27). Fucosterol and sistosterol have been identified. The lipid fraction of a *Scenedesmus* contained 18 per cent unsaponifiable material including large quantities of hydrocarbons and higher aliphatic alcohols; chondrillasterol was identified as the only sterol present in significant quantity (16).

The fats have not been shown to participate significantly in the metabolism of algae, as observed in their gas exchange, except in the dark respiration of diatoms (13). This problem should be investigated further in high-fat cells. Both qualitative and quantitative data indicate that the diatoms are not physiologically different from the Chlorophyceae in lipid formation (98, 102). The myth of fat production as a special characteristic of the diatoms probably has arisen from the low nitrogen content of their natural habitats and of the culture media frequently used.

Pigments.—A few observations should be added to supplement the reviews of Cook (32) and Strain (189). Chlorophyll analyses of 2.1 and 2.5 per cent of the dry weight and considerably lower values for carotenoid content of *Scenedesmus* and *Chlorella* are reported (31, 86); it is known, however, that the chlorophyll content varies widely with conditions of culture (88, 123). From measurements of the action spectrum of photosynthesis reasonable evidence has been presented for the participation of phycocyanin in the photosynthesis of the blue-green *Chroococcus* (47), of phycocyanin and phycoerythrin in a number of red algae (87), and of some of the carotenoid pigments in *Chlorella* (46).

Carbohydrates.—The variety of carbohydrates and carbohydrate-like materials formed by the algae have been covered by the review of Smith (184) [see also (92)]. It is significant that reducing sugars make up only a negligible fraction of the cell constituents. Sucrose has been identified in small amounts in *Scenedesmus* (23) and in *Chlorella pyrenoidosa* (121) although it will not support respiration or growth of the latter organism. It is also significant that the uronic acid polymers, such as alginic acid, which appear to be generally distributed, have pronounced ion exchange properties and may be of importance in ion accumulation.

Protein.—A high protein content is inferred from the high nitrogen

content of many algae. Only limited data are available on amino acid composition (49, 117, 118).

Other noteworthy constituents.—Substances of vitamin K activity have been demonstrated in *Chlorella* by Dam (39). Observation of widespread metabolic effects of such substances in *Chlorella* and *Scenedesmus* led Gaffron (68) to propose that the naturally occurring material may function as a metabolic catalyst or regulator.

The ascorbic acid problem merits further study. Algéus (3) made the conventional indophenol titration on metaphosphoric acid cell extracts of *Chlorella*, *Scenedesmus*, and *Stichococcus*. Extracts of autotrophically grown cells gave unreasonably high values of 1 to 5 per cent of the dry weight when the titration data were calculated in terms of ascorbic acid; however, dark-grown cells, even when used in greater quantities, yielded no titratable reducing activity. A stimulation of growth by ascorbic acid in the light was observed, even in the presence of added glucose. There are several possibly related observations. Gunderson & Skinner (80) failed to find ascorbic acid in dark-grown *Chlorococcum* using animal assay methods. Ondratschek (140) reported a stimulation of growth of *Euglena gracilis* and *Haematococcus* by ascorbic acid in the light but not in the dark; B_{12} was not specifically provided. Ascorbic acid has been shown to have a sparing effect on the B_{12} or thymidine requirement of a number of bacteria (cf. 103).

Economic Aspects

Noxious effects.—Algal blooms, particularly of blue-green forms, continue to present an economic problem in fresh water lakes and in water supplies (cf. 168). Blooms of marine dinoflagellates have been incriminated as the causes of mass mortality of fish (35, 81). A poison produced by the dinoflagellate *Gonyaulax catenella* and accumulated by shell fish has a remarkably high toxicity (175).

Sewage disposal.—The use of sewage lagoons or oxidation ponds has been proved an adequate and economical means of treatment of domestic sewage in Southern California and in Texas. Studies of Caldwell (25) and Myers (130) show that the typically profuse algal flora functions in maintaining dissolved oxygen concentrations as high as 300 per cent of air saturation during the day. By cyclic operation of algae and aerobic bacteria, the sewage organic matter is converted into algal cells. These may be disposed into natural streams in the effluent or allowed to sediment and digest anaerobically as a bottom sludge. The method has minimum odor nuisance and operating cost; it is ideally adapted to the needs of small communities in mild climates and to the collecting urge of the itinerant phycologist.

Mass culture of algae.—The culture of algae for the production of food and special organic materials has received considerable discussion (cf. 119) and a small amount of specifically directed experimental study. Pertinent to the problem is the work of Cook (33), Harder & von Witsch (83), Ketchum

et al. (101), Myers *et al.* (135), Spoehr & Milner (185), and von Witsch (206, 207). The essential problems, now definable, are to achieve maximum density of population, maximum rate of growth, and maximum light utilization for an alga whose cells contain materials of economic usefulness. Inadequate attention has been given to the requirement of high population density which is critical to the economics of harvesting procedures. After all, the plankton production of the oceans is great in magnitude but of too low a concentration to make harvesting feasible. Achievement of high population density requires the solution of special problems of nutrient provision and the use of very thin layers of suspension in order to afford adequate effective illumination to all the cells of the culture. A culture containing 55 gm. per liter dry weight produced at a rate of 2.5 gm. per liter per day has been reported for *Chlorella* (135).

The economic feasibility of algal mass culture is not here in question. However, it would be rather remarkable if the assembling of a body of knowledge on algal physiology, equivalent to that now available for other groups of microorganisms, would not reveal new ways of producing materials of economic usefulness.

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ENDOTOXINS[1]

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The nature of the toxicity of the cell substance of some pathogenic microorganisms, especially those that do not produce exotoxins, has been of perennial interest. While it can hardly be said that the pathogenesis of disease produced by these forms is adequately explained by the observed pharmacological activity of such substances, it is not unlikely that their liberation within the host through disintegration of the microbial cell contributes, at least in part, to disease. For present purposes the term endotoxin will be restricted to those toxic substances that are present in the intact bacterial cell, and differ in properties from the typical thermolabile, protein exotoxins. Endotoxin is most frequently found in the smooth, virulent forms of gram-negative bacteria, notably the enteric bacilli, the cholera vibrio, *Brucella*, *Pasteurella*, the meningococcus, and the gonococcus. This review is limited to a consideration of representative reports, largely of the past 20 years, concerned with the chemical nature, pharmacological activity, and antigenicity of the better known endotoxins.

Chemical Nature

Study of the toxic components of the cell substance is primarily dependent upon their separation from the bacterial cells by methods sufficiently mild that the material isolated is not significantly altered in the process. Disintegration of the cell and separation of toxic fractions is an obvious approach, and one that has been used by a number of workers. The method of tryptic digestion followed by alcohol precipitation of endotoxin described by Raistrick & Topley (1), and that of precipitation of endotoxin by 80 per cent saturation with ammonium sulfate from autolyzed cultures used by Prigge & Helmert (2) are representative of this method of isolation. Such studies have led to the characterization of endotoxins as heat stable, apparently nonprotein (but actually protein-containing) substances that are resistant to tryptic digestion but labile to mild acid hydrolysis, precipitable by alcohol or acetone or by the usual salting out procedures, and closely similar to, if not identical with, the somatic antigenic complex of the bacteria from which they are derived.

The solubility behavior of endotoxin activity has suggested differential extraction as a means of effecting preliminary isolation, and a number of such procedures have been devised. One of the first attempts to extract endotoxin selectively from intact cells was the trichloracetic acid method

[1] The survey of literature pertaining to this review was concluded in December, 1950.

developed in 1933 by Boivin and his co-workers (3, 4). The bacteria from 18- to 24-hour cultures were washed and suspended in distilled water in a concentration of about 200 mg. per ml. An equal volume of 0.5 N trichloracetic acid was added and the bacteria extracted for 3 hours at refrigerator temperature. The bacterial cells, morphologically intact and retaining their staining characteristics, were removed by centrifugation. The toxicity remaining in the supernate was nondialyzable, precipitated by 4 to 5 volumes of alcohol or acetone, gave an opalescent aqueous solution, and was negative to the usual qualitative tests for protein. It represented about 5 per cent of the dry weight of the bacteria, but the yield could be increased to about 10 per cent if the organisms were digested with trypsin prior to extraction. On intraperitoneal inoculation it killed 20 gm. mice in doses of about 0.1 mg. Toxicity was lost on hydrolysis in dilute acetic acid, and the residue was made up of 40 to 50 per cent polysaccharide and 20 per cent lipid; the remaining 30 per cent was not accounted for. It was concluded that the endotoxin or "antigène complet" was a glucolipid complex. The same kind of product was obtained from a number of different kinds of bacteria, including dysentery bacilli, *Salmonella*, the cholera vibrio, etc., in the smooth virulent form, but was found in only very small amounts in rough variants. In addition, similar endotoxins were extracted from such non-pathogens as the colon bacillus, *Proteus*, *Chromobacterium prodigiosum*, and *Phytomonas tumefaciens*. Subsequent extension of this procedure and comparison of the products isolated with endotoxins prepared by other methods have been reviewed by Boivin (5, 6, 7) and Mesrobeanu (8).

The Shiga dysentery bacillus.—The endotoxin of the Shiga dysentery bacillus was extracted by the trichloracetic acid method by Boivin and by a number of other workers of whom Haas (9, 10), Wagner-Jauregg & Helmert (11), and Boroff (12) are representative. Elucidation of the nature of the endotoxin was considerably furthered in a series of studies by Morgan (13) and Morgan & Partridge (14, 15). Specific polysaccharide, previously isolated by Morgan (16) and presumably the same as that occurring in the glucolipid complex described by Boivin, was found to be soluble in a number of anhydrous polyhydroxy organic solvents, including diethylene glycol, while a number of representative proteins were not. On the working hypothesis that the complex representing the endotoxin might also be soluble in such reagents to the exclusion of cellular protein, it was found that the endotoxin was slowly extracted from dry, intact bacterial cells in anhydrous diethylene glycol in four days, and that four successive extractions removed a total of about 10 per cent of the dry weight and all but negligible amounts of endotoxin. The solvent could be removed by dialysis to leave an opalescent aqueous solution, or the endotoxin precipitated from glycol with alcohol or acetone to give a white amorphous substance having a mouse lethal dose of about 0.1 mg. The material purified by precipitation represented 5 to 7 per cent of the bacteria and appeared closely similar to Boivin's preparations. Attempted fractionation with alcohol, acetone, or ammonium sulfate gave no indication that the material was a simple mixture of different substances,

and the contained lipid could not be extracted with ether, alcohol-ether, or chloroform, suggesting that it was an integral part of the complex.

The toxic complex could be broken down in a number of ways. On hydrolysis in dilute acetic acid the opalescence of the solution cleared, a fatty layer of phospholipid, insoluble in acetone but soluble in ether and alcohol-ether, separated on the surface, a precipitate of protein appeared, and the polysaccharide remained in solution. The lipid fraction was 9 to 12 per cent of the total, the protein fraction 17 to 20 per cent, and the polysaccharide fraction 55 to 60 per cent. While the protein in the original complex did not give the usual qualitative tests, thus accounting for Boivin's assumption that protein was not present, the residue did behave as a protein. When the complex was dissolved in cold anhydrous formamide, it dissociated into a protein-polysaccharide complex and a lipid, and, on the addition of alcohol to 75 per cent, the complex precipitated, leaving the lipid in solution. On continued treatment with formamide, the protein-polysaccharide complex also dissociated into its components. It was further found that primary extraction with 90 per cent phenol followed by alcohol precipitation gave the protein-polysaccharide complex free from lipid. When, however, the phenol was removed by dialysis, the complex was found to be dissociated and the protein precipitated, leaving the polysaccharide in solution. Essentially the same dissociation of the protein-polysaccharide complex occurred in dilute alkali in the cold.

The lipid component was made up of palmitic acid, oleic acid, α-glycerophosphoric acid, and a nitrogenous constituent that was not further investigated. The polysaccharide and protein components differed according to the method of their preparation. Acid hydrolysis produced a degraded form of polysaccharide which could not be recombined with the conjugated protein (see below), together with undegraded protein, while dissociation in phenol gave undegraded polysaccharide and a degraded protein. The main products of hydrolysis of the polysaccharide were N-acetylglucosamine, *d*-galactose, *l*-rhamnose, and possibly other sugars in small amount; the undegraded material contained about 1 per cent phosphorus, while the degraded form was phosphorus-free. The undegraded protein contained 11.5 to 12.5 per cent nitrogen and 0.6 to 0.8 per cent phosphorus, and was readily soluble in dilute alkali but insoluble in acid. The degraded protein contained 14.5 per cent nitrogen and no phosphorus, and the levorotation was increased over that of the undegraded protein. It was soluble between pH 2.0 and 2.5, and insoluble up to pH 7.0. It appeared, therefore, that the undegraded protein was a conjugated protein, differing from the degraded simple amphoteric protein in that it contained substances responsible for its acidic nature and low nitrogen and high phosphorus content.

Dissociation of the protein-polysaccharide complex in formamide was found to be reversible in that undegraded polysaccharide and conjugated protein recombined in formamide solution, and the complex, indistinguishable from that prepared by extraction of the bacteria, was precipitated on the addition of alcohol. Furthermore, artificial complexes between unde-

graded Shiga polysaccharide and certain acid-insoluble proteins, such as conjugated protein isolated from a glycol extract of the typhoid bacillus, could be prepared.

Morgan and his co-workers did not study the toxicity of these substances at any length, but in general toxicity appeared to be independent of the lipid component, and a property of the protein-polysaccharide complex. The toxicity of the components of the endotoxin complex was studied in detail by Tal & Olitsky (17). The diethylene glycol extract had a mouse lethal dose of about 1 mg. and the protein-polysaccharide complex freed from lipid by formamide dissociation was equally toxic. Of the individual components, only the conjugated protein was appreciably toxic with a lethal dose of 15 mg. Of the remainder of the activities studied, leucopenia was also produced by inoculation of the undegraded polysaccharide.

The Flexner dysentery bacilli.—The character of the endotoxin separable from Flexner dysentery bacilli has been examined in detail by Goebel and his co-workers (18, 19). It was poorly extracted by diethylene glycol, and that isolated from cells subjected to preliminary digestion with trypsin was reduced in protein content. Extraction in 50 per cent pyridine gave good yields and the endotoxin was separated from inert substances by fractionation on the basis of its solubility in 50 per cent acetone. A protein-acetyl-polysaccharide-lipid complex was isolated from types V, W, and Z and from the Newcastle bacillus that was lethal to mice in doses of 0.2 to 0.5 mg. In general, the endotoxin represented about 10 per cent of the dry weight of the bacteria. Treatment with strong alkali in the cold removed acetyl groups but did not affect toxicity. In alkaline alcohol the complex dissociated to lipid, nontoxic protein, and a toxic, deacetylated phosphorus-containing polysaccharide that was lethal to mice in doses of 0.5 mg. Hydrolysis of the toxic polysaccharide in dilute acetic acid resulted in loss of toxicity. On heating in saturated picric acid solution, the complex broke down to acetylated polysaccharide, lipid, and toxic protein. Treatment of the toxic protein with alkaline alcohol resulted in loss of toxicity and appearance of amphoteric protein. When the complex was treated with trypsin, protein was digested, with a reduction in nitrogen from 11.8 to 6.8 per cent and an increase in phosphorus from 1.3 to 2.1 per cent, and toxicity was not reduced. Thus toxicity was associated with organic phosphorus in combination with intact or partially hydrolyzed protein, or with polysaccharide. It was further shown by Tal & Goebel (20) that the product of pancreatic digestion of the complex was dissociated in acid formamide to give a substance retaining the original toxicity and containing 1.9 per cent nitrogen and 1.1 per cent phosphorus. It was treated with alkaline phosphatase and lipid removed by extraction with chloroform-methyl alcohol. The final product was toxic and contained 2.1 per cent nitrogen, only 0.2 per cent more than calculated for its glucosamine content. It appeared to be a phosphorylated polysaccharide containing about 1 per cent organically bound phosphorus. On hydrolysis in dilute acetic acid, toxicity was destroyed, a small amount of material high in carbon and phosphorus precipitated, leaving immunologically active,

nontoxic polysaccharide that differed from the parent substance in that it contained 1 per cent less carbon and 0.3 per cent less phosphorus. It was evident that the toxicity of the endotoxin was not attributable to the lipid, protein, or polysaccharide components of the complex, but to a phosphorus-containing substance as yet not characterized, and probably the precursor of the insoluble material split off by hydrolysis.

Other dysentery bacilli.—The endotoxins of dysentery bacilli other than Shiga's bacillus and those of the Flexner group have been studied only superficially. Trichloroacetic acid extracts of intact bacilli or autolyzed broth cultures of the Schmitz bacillus have been prepared by Haas (21), Schröer (22), and Buchwald (23). The endotoxin so extracted was lethal for mice and appeared to be closely similar to Boivin's preparations, containing polysaccharide and giving negative tests for protein. A similar toxic extract of the Sonne dysentery bacillus was prepared by Roelcke & Berdan (24) who reported that it consisted of polysaccharide plus inorganic phosphate. Trichloroacetic acid extracts of *Shigella alkalescens* were studied by Edward (25) who concluded that the endotoxin so prepared was a glucolipid complex. Relevant literature on the dysentery bacilli has been summarized by Weil (26, 27).

Salmonella.—Considerably less definitive information is available concerning the nature of the endotoxins of bacteria of the Salmonella group than those of the dysentery bacilli. Trichloroacetic acid extracts of *S. typhi-murium* (*aertrycke*) and *S. enteritidis* were prepared by Boivin & Mesrobeanu (3, 4), and similar preparations of Vi-containing and W strains of the typhoid bacillus were studied by these workers (28, 29). The activity was associated with a complex giving strong Molisch and negative protein tests, and polysaccharide and lipid were isolated following hydrolysis. The complexes were toxic to about the same degree as those isolated from dysentery bacilli and appeared to be closely similar substances. It was found possible to separate the complex extracted from Vi-containing typhoid bacilli into components representing the immunological activity of the O and Vi antigens by precipitation of the latter with alum, uranyl acetate, or phosphotungstic acid; the separated Vi complex was somewhat less toxic than the O complex. Haas (30) prepared endotoxin by trichloroacetic acid extraction of species of groups B and D, and showed cross neutralization associated with the common antigen XII; for instance, antiendotoxic typhoid sera neutralized paratyphoid B endotoxin and vice versa.

The endotoxin of *S. typhi-murium* was prepared by Raistrick & Topley (1) from a tryptic digest of acetone-killed bacteria by precipitation at 68 per cent (by weight) alcohol. The product represented about 10 per cent of the dry weight of the bacteria. The toxicity of their preparations was studied by Martin (31); they were lethal to mice in doses of 0.1 to 0.5 mg., and toxicity was destroyed by mild acid or alkaline hydrolysis, during which the complex broke down to polysaccharide and lipid plus some nitrogenous material. Using the method of tryptic digestion, Topley, Raistrick, Wilson, Stacy, Challinor & Clark (32) isolated endotoxin from a Vi-containing strain

of the typhoid bacillus and strain 901, a W strain. Neither gave any precipitate with reagents such as sulfosalicylic acid, trichloroacetic acid, silver nitrate, and mercuric chloride, but did give positive Millon, biuret, ninhydrin, and Molisch tests. Only the Vi-containing preparation gave precipitates with phosphotungstic acid in 5 per cent sulfuric acid, neutral lead acetate, uranyl acetate, mercuric acetate and aluminum sulfate.

The method of extraction in diethylene glycol was applied to O, Vi, and rough Vi strains of the typhoid bacillus by Henderson & Morgan (33). The endotoxins were precipitated from the extract by acetone or alcohol. The substances so isolated were very similar to those prepared by trichloroacetic acid extraction or tryptic digestion, and about equally toxic. The mouse lethal dose of the O and O plus Vi endotoxins was 0.2 to 0.3 mg., but the Vi alone from the rough strain was less toxic and the lethal dose was about 5.0 mg. The preparations did not give protein tests such as precipitation with trichloroacetic or sulfosalicylic acids, but it was observed that the O and O plus Vi endotoxins gave a positive ninhydrin test, while the Vi preparation did not. Precipitation of the Vi preparation by uranyl acetate, etc., noted above was observed also.

Extraction of the endotoxins of *S. typhi-murium* and *S. typhi* by 2.5 and 6.5 *M* urea was described by Walker (34). The endotoxins precipitated from the cell-free extracts with 68 per cent alcohol were polysaccharide-containing amorphous substances that were lethal for mice in doses of 0.2 mg. for the typhoid bacillus and 0.4 mg. for the mouse typhoid bacillus. They did not differ significantly from endotoxins prepared by other methods.

Freeman, Challinor & Wilson (35) prepared endotoxin from *S. typhi-murium* and *S. typhi* by three of the methods referred to above, i.e., trichloroacetic acid extraction, tryptic digestion, and diethylene glycol extraction of cultures grown on synthetic medium, "equalizing" the procedures by precipitation of the activity at 68 per cent alcohol. The products differed from one another in only minor respects and were regarded as essentially identical. They had the same properties and toxicity as the preparations of other workers described above.

Morgan (36, 37) precipitated liquid cultures of the typhoid bacillus grown in semisynthetic medium with alcohol in the presence of sodium acetate. The precipitate was extracted with distilled water, the extract dialyzed, and the activity purified by successive precipitations with dilute acetic acid. It was similar to the preparations described above, but slightly less toxic, possibly as a consequence of the presence of Vi antigen.

To this point endotoxin preparations from Salmonella were regarded as glucolipid complexes, and protein-free on the basis of negative reactions of the purified complexes to the usual qualitative tests. Following the demonstration by Morgan & Partridge (15) of the protein moiety in the endotoxin of the typhoid bacillus as well as the Shiga dysentery bacillus, Freeman & Anderson (38) studied the products of mild acid hydrolysis of the complex isolated from the typhoid bacillus. They found it to consist of 57 per cent polysaccharide, 16 per cent acidic polypeptide, 10 to 20 per cent of an

alcohol-soluble nitrogenous substance, and 3 to 4 per cent lipid. The polysaccharide was acetylated and on hydrolysis gave *d*-mannose, *d*-galactose, and *d*-glucose. The polypeptide component contained 8.2 per cent nitrogen and 2.4 per cent phosphorus, and the lipid fraction consisted of a mixture of fatty acids and phospholipin.

The cholera vibrio.—The endotoxin of the cholera vibrio was one of the first to be studied intensively. The microorganism is relatively fragile and breaks up readily to liberate endotoxin, simulating exotoxin formation. It was early established by Pfeiffer that the toxicity does not diffuse out of the intact cell; the relevant literature is summarized by Bernard & Gallut (39). It was subsequently shown by Boivin & Mesrobeanu (3, 4) that the endotoxin could be extracted with trichloroacetic acid and is apparently of glucolipid nature. Extracts of this kind were studied by Raynal, Lieou & Feissole (40, 41) who reported that the toxin equivalent of 5 mg. of vibrios was the lethal dose for the mouse; this compares with the equivalent of 1 to 5 mg. of dysentery bacilli, and in general the cholera vibrio is less toxic per unit weight of intact organisms than are the dysentery bacilli. Gallut (42) found, however, that relatively small quantities of toxin are extractable from the vibrios, reporting yields of 1.8 to 4.4 per cent, but that the lethal dose for the mouse was about 0.05 mg. This indicates that, while the amount of toxin in the individual cell is less than in the dysentery bacilli, it has greater activity; the methods of assay of both content of toxin and toxicity are only roughly quantitative. Bernard & Gallut (43, 44) showed that maximal yields of endotoxin could be obtained by incubation for 4 hr. in glucose broth, centrifuging, adjusting the pH, and reinoculating the supernate, the process being repeated three or four times. Endotoxin diffused from the organisms killed by the developing acidity in the successive cultures, and accumulated in the medium. The endotoxin was studied in detail by Gallut & Grabar (45 to 49) who have shown that it contains two substances, an antigenic thermostable glucolipid, and a nonantigenic thermolabile protein-like substance characterized by its hypothermic activity.

The cholera endotoxin was isolated by Burrows (50) from cells ground with sand, dissolved in 6 *M* urea, or digested with trypsin, and from intact cells by extraction with 0.5 *M* trichloroacetic acid, methyl or ethyl alcohol, chloroform or ether, but it could not be extracted in glycols. Polysaccharide was precipitated from trichloroacetic acid extracts by 3 to 5 volumes of alcohol. The toxicity remained in solution; it dialyzed through cellophane and so could not be separated from trichloroacetate in this way. Dialyzability of the activity has been reported by a number of other workers, such as Basu, Chaudhury & Basu (51). On concentration of the alcohol-precipitated trichloroacetate solution, the toxicity separated in a yellow oil. It could also be prepared by alcohol precipitation of ground cells, urea solution, or digested cells, and by direct extraction of the intact cells with methyl or ethyl alcohol, or chloroform, but was only slowly extracted in ethyl ether. It was precipitated from alcohol solution by the addition of 10 volumes of chilled acetone to give a white amorphous lipid substance that was negative

to Molisch, Millon, and biuret tests, and contained 5 per cent nitrogen and 1.7 per cent phosphorus. The activity was heat stable, but unstable to 0.1 *N* NaOH at room temperature and the purified substance was insoluble in water, precipitating when the alcohol concentration was reduced to 20 per cent. It was inactive in aqueous suspension, but the activity was restored on re-solution in alcohol. The mouse lethal dose was 0.03 mg., slightly smaller than that observed by Gallut (42).

The cholera vibrio has a marked tendency to spontaneous lysis, and in studies on phosphorus distribution following immune lysis of this organism the reviewer has observed a rapid shift in lysate phosphorus from the nucleoprotein and phospholipid fractions to the acid soluble fraction. It is not unlikely that the endotoxin complex may break down readily with toxicity persisting in some portion or portions of the residue. Instability of the intracellular endotoxin complexes of other organisms such as the dysentery bacilli has been emphasized by many workers, and, in fact, Boivin & Mesrobeanu (52) have shown that *S. typhi-murium* and *S. enteritidis* contain an enzyme that breaks down the endotoxin complex.

Other bacteria.—A number of other gram-negative bacteria contain endotoxins, and preparations of them similar to those described above have been studied. Pirosky (53, 54, 55) prepared trichloroacetic acid extracts of *Pasteurella aviseptica* that appeared to be glucolipid in nature, and killed mice in doses of 0.4 to 0.8 mg. Boor & Miller (56, 57) found that endotoxin could be extracted from meningococcus and gonococcus in trichloroacetic acid, 0.5 *N* HCl, or diethylene glycol, and from a "nucleoprotein" fraction soluble in dilute alkali by trichloroacetic acid. The substance killed mice in doses of 0.2 mg., was glucolipid in nature, and the toxicity was stable to heat and tryptic digestion.

Chemical fractionation of *Brucella* has been carried to considerable length; the literature is reviewed by Pennell (58) and need be considered only briefly here. Pennell & Huddleson (59) prepared a toxic fraction they termed "endoantigen" by tryptic digestion of dried ground cells, followed by extraction with 0.25 *N* trichloroacetic acid and precipitation of the activity with alcohol. The preparations contained lipid, gave reducing substances on acid hydrolysis, and had a total nitrogen content of 6 to 9 per cent. Trichloroacetic acid extracts of intact cells were prepared by Pop and his co-workers (60, 61, 62) that represented 4 to 6 per cent of the dry weight of the bacteria, contained 4 to 5 per cent nitrogen, 12 to 20 per cent lipid, 10 to 16 per cent reducing substance on hydrolysis, and killed mice in doses of 0.1 mg. Miles & Pirie (63 to 67) prepared a "native antigen" from an autolysate of phenol-killed bacteria by ammonium sulfate precipitation and centrifugal fractionation of an aqueous solution of the precipitate; the active complex sedimented in 30 min. at 14,000 r.p.m. in a 15 cm. rotor. The mouse lethal dose of this preparation was about 5 mg. in contrast to 11 mg. for intact bacteria or tryptic digest of bacteria, and it contained 4 to 5 per cent nitrogen. Lipid to about 20 per cent could be extracted with acidified alcohol-ether, reducing the lethal dose to about 2 mg. An inactive polypeptide frac-

tion was removed by extraction with 80 per cent acetic acid and precipitation with 1.3 volumes of alcohol. The remaining complex, designated amino polyhydroxy antigen, was lethal to mice in doses of 0.1 to 0.2 mg. and regarded as analogous to the endotoxin complex of enteric bacilli described above. Toxicity was rapidly lost on hydrolysis in dilute acid, and the decomposition products were phospholipid, a formyl amine, and free amine, the last representing an amino sugar converted to a non-reducing anhydride during hydrolysis.

Pharmacological Activity

The toxin action of the bacterial endotoxins has been noted casually by many workers and studied intensively by some. Delaunay & Lebrun (68) have summarized the literature to 1947. They point out that a variety of animals are susceptible to the action of the bacterial endotoxins including, in addition to man, the mouse, rat, rabbit, sheep, goat, horse, dog, pig, and a number of birds, while in general the cold-blooded animals are not affected. The commonly used experimental animals are the mouse and rabbit; the latter is frequently the more susceptible on a body weight basis by a factor of 5 to 100, but somewhat irregular in response, while the mouse is relatively uniformly susceptible to a median lethal dose of 0.1 to 0.5 mg. of purified material. The rat is considerably more resistant, and the median lethal dose is 6 to 8 mg. In man an intradermal dose of 0.001 to 0.0001 mg. is usually sufficient to give a marked local reaction and sometimes a general one.

The action of the endotoxin of *S. typhi-murium* has been studied in detail by Delafield (69) and Cameron, Delafield & Wilson (70); that of the dysentery bacilli by Olitski, Avinery & Bendersky (71) and Koch & Olitski (72); that of the typhoid bacillus in experimental animals by Martin (31) and Morgan (73 to 76) and in man by Favorite & Morgan (77); and that of the cholera vibrio by Bernard & Gallut (39). Pirosky (53) has described the action of *Pasteurella aviseptica* endotoxin in mice, and Pop, Damboviceanu, Barber & Marinov (60) and Miles & Pirie (65) that of *Brucella* endotoxin. The striking feature of these and related studies is the close similarity in dosage, symptoms, and post mortem findings regardless of the species of bacteria from which the endotoxin is derived. When the inevitable subjective element is taken into consideration, it seems probable that the toxic action of endotoxins of diverse origin and methods of preparation is substantially identical, and may be considered as a whole.

In the experimental animal the incubation time is short, almost always less than an hour, and the symptoms reach a peak in 2 to 8 hr. These include dyspnoea, tremors, diarrhea, sometimes prostration and paraplegia, leucopenia, and an early hyperglycemia succeeded by a hypoglycemia that often reaches convulsive levels in fatal intoxication. The majority of animals receiving lethal doses die within 24 hr., but recovery from sublethal doses is rapid. At autopsy the small intestine is hemorrhagic and distended with yellow fluid containing desquamated epithelial cells, and Peyer's patches are markedly congested. The spleen, liver, and kidneys may be edematous,

congested, and often hemorrhagic; the adrenals, pancreas, and thyroid show evidence of hemorrhagic congestion; and there are foci of edema, congestion, and hemorrhage in the lungs. The lymphatic tissue, heart muscle, brain and spinal cord, and bone marrow show similar pathology, and in general thrombosis and vascular damage are widespread.

The mechanism of endotoxin action.—The observed effects of the endotoxins have been taken to suggest (*a*) that these substances are primarily vascular poisons, (*b*) that the effect is an indirect humoral one, and (*c*) that direct action of the endotoxin is on the nervous system. The first of these viewpoints has been emphasized by Zahl and his co-workers (78 to 82) in their studies on the induction of hemorrhage in tumor tissue and decidual placenta by sublethal doses of endotoxins from a variety of gram-negative bacteria. They are of the opinion that the primary effect of the endotoxins is on the vascular system. This view is perhaps partially supported by the observations of Delaunay and his co-workers (83 to 86) who have shown that the first stage of intoxication is a series of constrictions and dilatations of the small arteries and arterioles (observed in the rabbit ear) that was not suppressed by extirpation of the cervical sympathetic ganglion. According to these workers, the cellular lesions resulting from repeated sublethal doses of endotoxin may be regarded as having their origin in vascular disturbances. Further examination of the circulatory changes in the living animal, however, showed that they resemble those of traumatic shock, i.e., increased rate and decreased force of heart beat, peripheral circulatory failure, increased resistance of the capillaries to rupture from externally applied pressure, etc., suggesting a humoral or neurogenic reaction to the endotoxin. Perhaps complementary to these observations are those of Kun (87) who has shown that meningococcal endotoxin markedly reduces blood histamine and increases tissue histamine in the rabbit, and that death is accelerated by the simultaneous inoculation of histamine.

The consistent effect on blood sugar, a hyperglycemia followed by a hypoglycemia, of endotoxin toxemia is suggestive of an impairment of carbohydrate metabolism. The effect of toxemia on metabolism has been reviewed by Holmes (88). He summarizes strong experimental evidence supporting the conclusion that the metabolism of three and four carbon compounds (thus including fat and protein metabolism) is affected. This point has been studied in some detail by Kun and his co-workers. Kun & Miller (89) showed that in the hypoglycemic stage of intoxication with meningococcus and *S. typhi-murium* endotoxins, blood and tissue concentrations of lactate were increased, while that of pyruvate was decreased, indicating tissue anoxia and consequent inhibition of the adenylic system. *In vitro* experiments showed that succinic-dehydrogenase was markedly inhibited by endotoxin, while cytochrome oxidase activity was not affected. They also found that the liver was almost completely exhausted of glycogen, and similar depletion of liver glycogen in the rat following inoculation with Shiga endotoxin has been reported by Tal & Olitski (17). Kun (90) further showed that *S. typhi-murium* endotoxin inhibited glycogen formation from

pyruvate and glucose *in vivo* and *in vitro*, and Kun & Abood (91) found that pyruvate oxidation was also inhibited both *in vivo* and *in vitro* by the endotoxin.

There is also some evidence that may be construed as supporting a direct effect of endotoxin on the nervous system. That reviewed by Delaunay & Lebrun (83) includes the production of symptoms of endotoxin intoxication by direct inoculation of the splanchnic nerve or mesenteric ganglion, or by intraventricular inoculation. It is difficult to rule out dissemination of the endotoxin from the site of inoculation in such experiments, and the evidence cannot be regarded as unequivocal.

The effect of chemotherapeutic drugs.—In conjunction with studies on the mechanism of action of chemotherapeutic drugs, the effect of these substances on the endotoxins has been of interest to a number of investigators. Levaditi & Vaisman (92) studied the protective effect of sulfanilamide, 4-nitro-4′-amino diphenyl sulfoxide, 4-nitro-4′-amino diphenyl sulfone, and sodium 4′-sulfamido phenylazo-3-5-diamino benzene against saline extract endotoxins of the gonococcus, meningococcus, and *P. avicida* (*aviseptica*), and trichloroacetic acid extracts of *S. typhi-murium* and Shiga and Flexner dysentery bacilli. Mice which received endotoxin alone showed 90 to 100 per cent mortality. With the same doses of toxin, sulfanilamide, the sulfoxide, and sulfone decreased the mortality markedly, occasionally to 20 per cent. The azo compound was not effective. Carpenter, Hawley & Barbour (93) also showed that sulfanilamide protected to a small degree against the toxicity of broth culture of the gonococcus. Zahl & Hutner (94) found that tumor-bearing mice could be protected against 16 minimum hemorrhage doses of *S. typhi-murium* endotoxin with sulfanilamide, and reported too (95, 96) that compounds of the sulfonamide series protected against 2 to 10 lethal doses of the endotoxin. Miller and his co-workers have shown that penicillin protects against 1 to 2 lethal doses of gonococcal (97) or meningococcal (98) endotoxin, and against as much as 9 lethal doses of *S. typhi-murium* endotoxin (99) when impure penicillin was used. There is no apparent neutralization of endotoxin by these substances *in vitro* and protection conferred by prophylactic sulfanilamide is highly transitory (92). On the basis of highly exploratory experiments in which hydroquinone, acetyl hydroquinone, and 4-4′-dioxyazobenzene showed some activity against gonococcus and meningococcus endotoxins, Levaditi & Vaisman (92) suggested that endotoxin neutralization is a consequence of an *in vivo* oxidation-reduction reaction.

Immunological Activity

Essentially without exception the trichloroacetic acid extracts, or Boivin antigens, have been found to be complete antigens and, among the enteric bacilli at least, identical with the somatic antigen (7). The endotoxin complex extracted or separated from the bacterial cell substance by the variety of other methods discussed above have likewise been shown to function as complete antigens, eliciting the formation of agglutinating, precipitating,

antitoxic, and protective antibody. The complex extracted in diethylene glycol by Morgan (13) from the Shiga dysentery bacillus was found to be a potent antigen; 0.3 mg. in 6 doses gave high titer agglutinating antisera, and 0.015 mg. of acetone-precipitated endotoxin given in 3 doses was equally effective. The protein-polysaccharide complex separated from the lipid component was shown by Morgan & Partridge (15, 100) to give a good immune response as measured by agglutinin titer. On further degradation antigenicity was lost, the polysaccharide fraction functioned as a haptene, and on reconstitution of the complex in formamide antigenicity was restored. The specificity of the polysaccharide was further underlined by the Shiga specificity of Shiga polysaccharide coupled with acidic protein moiety of the typhoid bacillus. Morgan & Schütze (101) and Schütze (102) found that the protein-polysaccharide complex, prepared by acetone precipitation of phenol extract, is highly effective in man, producing high titer protective antibody.

Similarly, Goebel and his co-workers (18, 19) found that the endotoxin complex of the Flexner dysentery bacilli is a complete antigen, and this substance and acetylated polysaccharide reacted to high titer with antiserum to whole organism, while the other degradation products reacted only weakly or not at all. Subsequently, Perlman, Binkley & Goebel (103) showed that the acetylated polysaccharide, like that of the pneumococcus, is weakly antigenic in man though not in experimental animals. The identity of agglutinating, precipitating, and antitoxic antibodies with one another and with antibody to the toxic protein-polysaccharide complex was shown by the detailed quantitative studies of Perlman and Goebel (104); they also found that cross reactions among the Flexner bacilli are due to similarities in the polysaccharide haptene rather than shared identical polysaccharides (105).

The observations of Henderson & Morgan (33), Walker (34), Freeman and his colleagues (35, 38), Topley *et al.* (32), and others have shown that the endotoxin complexes isolated from bacteria of the salmonella group are also antigenic while the degradation products are not, and the polysaccharide moiety functions as a haptene. The bulk of the relevant literature is summarized by Boivin (106). The endotoxin complex appears to be identical with the somatic antigen, but there is not sufficient precise work analogous to that of Perlman & Goebel (105) with the Flexner dysentery bacilli to show whether the observed immunological heterogeneity is attributable to a group of polysaccharide haptenes in a single complex, i.e., shared antigens, or to cross reactions between individual similar polysaccharides. In the case of the typhoid bacillus, however, the O and Vi antigens are biochemically as well as immunologically differentiable as described earlier.

It is commonly said that the endotoxins are poor antigens, and stimulate antibody production only to low titers. Examination of the relevant literature shows that this is not generally true so far as agglutinogen and precipitinogen are concerned, for relatively small doses of purified endotoxin produce high titer antibody. Henderson & Morgan (33) reported, for example,

that a total of 26 mg. of typhoid bacillus endotoxin produced agglutinin titers in the horse of 1:50,000, and 60 mg. produced a titer of 1:100,000; and these sera precipitated as little as 0.001 mg. of antigen. Similarly, Goebel and his co-workers found that a total of 0.15 mg. of Flexner type V endotoxin gave agglutinin titers of 1:3200 in the rabbit (18), and precipitated in a dilution of 1:100,000 in antibacterial sera (103).

The endotoxins also produce immunity to infection equivalent to that given by intact bacilli. For instance, Pirosky (54) reported that active immunization of mice with *P. aviseptica* endotoxin protected against 1000 or more median lethal doses of bacilli; Perlman, Binkley & Goebel (103) found that a 10-fold increase in protective antibody titer was produced in human volunteers immunized with 0.022 to 0.100 mg. of Flexner type V endotoxin; Henderson & Morgan (33), Topley *et al.* (32), Walker (34), Freeman, Challinor & Wilson (35) and others have reported similar observations with endotoxin of the typhoid bacillus and other species of *Salmonella;* and Burrows, Mather, Wagner & McGann (107) found that a total of 0.234 mg. of cholera endotoxin protected mice against 10,000 median lethal doses of vibrios. Exception may be noted in the case of gonococcus and meningococcus endotoxins; according to Boor & Miller (57), protective immunity is produced only irregularly.

The poor reputation of the endotoxins as antigens rests largely on their inability to stimulate the production of high titer antitoxic sera; even though agglutinin and precipitin titers are high, antiserum will neutralize only a few lethal doses of endotoxin. Much of the earlier work has been summarized by Boivin & Mesrobeanu (108, 109). Pirosky (53, 54), for example, could protect against only four to eight lethal doses of *P. aviseptica* endotoxin; Perlman & Goebel (104) against ten lethal doses of Flexner type V endotoxin; and Burrows *et al.* (107) could not demonstrate appreciable protection against cholera endotoxin. Similarly, Morgan (74) found that the leucopenia produced in rabbits by typhoid bacillus endotoxin was only somewhat less marked in the immune than in the normal animal, and Zahl, Hutner & Cooper (110) reported that immunized tumor-bearing mice were resistant to only about 200 minimum hemorrhage doses of endotoxin.

The apparent discrepancy between high precipitin titer, for instance, and low antitoxic activity may be explained by the observations of Morgan. He found (74) that when endotoxin was completely precipitated by antibody at optimal proportion, no toxicity remained in the supernate, but the washed precipitate was still toxic. He has further shown (111, 112) that the febrile and other toxic symptoms produced in rabbits and in man by inoculation of typhoid bacillus endotoxin are reduced by repeated inoculations with this substance, but because the moderately refractory state was not associated with antibody titer, he suggested that it may be a tolerance rather than the manifestation of an antibody response.

Summary

While evidence is still far from complete, that available, in the opinion of the reviewer, supports the following tentative conclusions. The bacterial

endotoxins make up a relatively homogeneous group of substances that exist as protein-polysaccharide-lipid complexes in the cell. Of these components, the lipid is pharmacologically and immunologically inert. The polysaccharide, which may or may not be esterified (acetyl, formyl), functions as a haptene and is responsible for the immunological specificity of the complex. The protein moiety is an acidic conjugated protein, but not a nucleoprotein, and in combination with the polysaccharide does not give the ordinary qualitative tests for protein and is stable to heat and proteolytic enzymes. Toxicity is associated with a phosphorus-containing nitrogenous substance that makes up a relatively small part of the complex, is usually bound to the protein but may occur in combination with the polysaccharide, and is active only in combination. The mode of action of the toxic complex is as yet not clearly defined, but it appears to be a vascular poison that also affects the metabolism of carbon compounds by inhibition of pyruvate metabolism. The endotoxin complex is highly antigenic but the toxicity is not effectively neutralized by antibody. In this respect it resembles certain naturally-occurring substances such as enzymes, viz., catalase, and the artificially prepared pharmacologically active azo antigens which stimulate antibody formation and react *in vitro* with homologous antibody, but are not neutralized by antibody. It then follows that effective immunity to the diseases caused by endotoxin-containing bacteria is primarily an antibacterial immunity that prevents endotoxin formation by direct action on the microorganisms, and in which the removal of toxicity is essentially incidental to the antigen-antibody reaction.

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ANTIBIOTICS[1]

With Particular Reference to Mode of Action

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Introduction

Definitions.—This review will be concerned with the methods by which naturally occurring substances interfere with the vital nutrition of microorganisms, and synthetic compounds are considered only where they contribute to the argument. Interest in these delicate and often specific poisons chiefly arose from their successful application as chemotherapeutic drugs. Their mode of action, even when unknown, must condition their effective application. For example, the influence of numbers of parasites, of concentration of agent, of time of exposure, and whether the enzyme system which is damaged is immediately vital or concerns the longer term function of cell-division, feature as practical problems in the emergence of resistant strains and, possibly, in changes in resistance of organisms.

Again, precise knowledge of the mechanism of interference might be expected to unmask the nature of the active groupings, and so point the way to synthetic modifications, as indeed have the studies of the mode of action of penicillin by Gale & Taylor (1) and Hotchkiss (2) thrown light upon the more general understanding of the biochemistry of bacterial protein synthesis.

Terminology: the meaning of "action."—The vital nutrition of the microorganism may be deranged on different metabolic planes. Should the enzymatic processes of respiration be affected, death is immediate; on the other hand, should less immediately vital systems be disorganised, cell-division may become impossible. Alternatively, the metabolism of the cell may gradually come to a standstill because essential syntheses take unnatural directions. In this simplified view, the rate at which the vital processes are moving govern which end-point is recorded by the observer. Usually, the crude observation of the effect of poisons on microorganisms has been that of sudden death (spoken of as a bactericidal action), or prevention of multiplication (usually referred to as bacteriostasis). These traditional and imprecise terms have many shades of meaning. A number of observations on the mode of action of antibiotics have been described in these terms and are of limited value. Rahn's (3) comprehensive review may be quoted as an example of several which stress the essentially quantitative nature of estimates of bacteriostasis.

The action of many antibacterial substances may be modified or inhibited

[1] The survey of literature for this review was concluded in March, 1951.

("reversed") by other substances. In this way, the restoration of some or all of the biological functions of a bacterial cell is possible after the "death" of the cell, when judged by other standards. Examples of reversibility given by Klarmann (4) are (*a*) desorption or elution of adsorbed antiseptics; (*b*) neutralization of acid by alkali; (*c*) inactivation of formaldehyde by ammonia; (*d*) inactivating of antiseptic chelating substances by metal ions; (*e*) inactivation of mercurials and other metallic compounds by thiols and of antibacterial thio compounds by metal salts; (*f*) impairment of the action of antiseptic dyes by hydrogen ion variation or ionic displacement; (*g*) reaction between cations and phospholipids; (*h*) impairment of oxidizing agents by reducing substances; (*i*) antagonism between sulphonamides and *p*-aminobenzoic acid (and allied substances); and (*j*) impairment of the action of antibacterial metabolite-analogues by essential metabolites. Additional examples are the reversal of streptomycin by lipositol and the destruction of penicillin by penicillinase.

Penicillin

Penicillin is the most effective antibiotic yet known. It has been the subject of detailed and repeated enquiry and will serve as a basis for comparison of others. It is an acid, the most acceptable formula for which may be represented in Fig. 1.

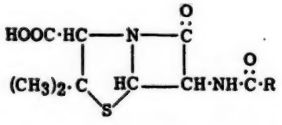

Fig. 1. General formula of the penicillic acids. Several penicillins exist: all are strongly acidic dipeptides having the molecular formula $C_9H_{11}O_4N_2SR$, the difference between them being in the constitution of the radical R. Salts of sodium, calcium, and potassium are used. The variations of R are the following:

Penicillin-I (F): R is $—Ch_2 \cdot CH=CH \cdot CH_2 \cdot CH_3$ (Δ^2-pentenyl)
Penicillin-II (G): R is $—CH_2 \cdot C_6H_5$ (benzyl)
Penicillin-III (X): R is $—CH_2 \cdot C_6H_4 \cdot OH$ (*p*-hydroxybenzyl)
Penicillin-IV (K): R is $—CH_2 \cdot (CH_2)_5CH_3$ (*n*-heptyl)
Penicillin Dihydro-F: R is $—CH_2 \cdot (CH_2)_3 \cdot CH_3$ (pentyl)

Molecular weights of the sodium salts have been estimated from freezing point determinations and by measurement of the diffusion constants [Klenlow (5)]. The estimates vary between 312 and 490. Kumler & Alpen (6) find these observations consistent with the behaviour of a completely dissociated electrolyte.

Significance of physical factors.—In reviewing the known facts about the various physical measurements made with penicillin and streptomycin, Hauser (7) concludes that these antibiotics are colloids and that their antibiotic activity depends upon their colloidal properties. The precise signifi-

cance of those predominating physico-chemical characters which determine the adsorption of antibiotics to microorganisms is difficult to assess, but clearly, they need not be identical with the "active groups" which determine selective toxicity. Dyestuffs which are strongly adsorbed, in the sense in which dyes are adsorbed to natural fibres, need not be antibiotics; yet antibiotics which are strongly adsorbed may well be more effective than those which are not.

Hauser (7) has demonstrated bacterial adsorption of penicillin. A liquid culture of *Staphylococcus aureus* observed at suitable magnification does not fluoresce in ultraviolet light. Benzyl-penicillin does. It is blue. Should the two be brought together, the fluorescence becomes concentrated on the surface of the cells and on the point of contact between cell and cell. As the fluorescence at these interfaces increases so is the fluorescence of the continuous phase decreased. In this way, it is shown that the dispersed phase of the penicillin sol is concentrated, in the first instance, at the interfaces.

Estimates of the amount of penicillin taken up by bacteria seem to show that this is similar in sensitive and insensitive forms, and in living and dead organisms. Rowley, Cooper, Roberts & Lester Smith (8) observed the radioactive penicillin uptake of four penicillin-sensitive strains of staphylococci compared with that of two resistant genera, *Escherichia coli* and *Klebsiella pneumoniae*. None produced penicillinase. When a thick suspension containing few or no dividing cells was observed, the penicillin uptake appeared to vary directly with the concentration of penicillin used. With low concentrations of less than 0.1 unit per ml., the ultimate concentration found inside sensitive organisms was much greater than that in the external environment, but with insensitive organisms it was the same. When cell-division was rapid, even resistant organisms took up penicillin, and the amount increased the greater the sensitivity of the strain. Resting cultures of sensitive staphylococci took up as much penicillin in 3 min. as they ever did; rapidly dividing cultures, on the other hand, showed an initial rapid uptake and a final value of ten times the resting cell amount. The maximum value was reached shortly before growth ceased. Finally sensitive strains killed by pretreatment for 30 min. at 37° C. with formaldehyde, cetyltetra-ammonium bromide, acriflavine or hydrochloric acid took up penicillin just as resting cells did; however, pretreatment with penicillin or buffered acetic anhydride prevented the expected uptake. The authors believed that a direct chemical reaction was involved between a cell substance and penicillin. It seems, however, that the observations do not appear to rule out adsorption of the kind typified by activated charcoal. Again, no vital (enzyme) system appears to be involved. However, the reacting substance may be a product of cell (division) nutrition and probably sensitive cells contain more of it than do insensitive cells.

Let us now examine the assumption that growth is essential for the demonstration of penicillin action. The earliest fundamental observations of Chain & Duthie (9), Hobby, Meyer & Chaffee (10), and of many others

showed that penicillin acts on susceptible bacteria when they are growing but not when they are in a resting phase. Its action is influenced only slightly by the number of organisms present. It is specifically inhibited by penicillinase, an enzyme which destroys penicillin. Early studies showed that with high concentrations of penicillin the number of organisms decreased at a constant rate until 99 per cent of the organisms had been destroyed. The rate of loss of vitality depends on the penicillin concentration up to an optimal amount of about 2 I.U. per ml. This effect may be demonstrated by transfer to an environment suitable for growth but now containing no penicillin. Quite a different impression is made by observing visible turbidity or the absence of it after 24 hr. exposure to a few hundredths of a unit of penicillin per ml. A test of this kind ignores invisible multiplication, or proliferation followed by lysis or loss of vitality. The effect of penicillin on metabolic processes other than visible growth had already been reported, in that Chain & Duthie (9), Schuler (11), and Dufrenoy & Pratt (12) had used differences in respiratory changes as a measure of failure to grow in a bacterial culture. Until 1946 there appears to have been but a single demonstration that penicillin could affect the metabolic system in anything but a growing cell. This was the observation of Krampitz & Werkman (13) that the slow breakdown of ribonucleic acid by starved washed bacteria was prevented by penicillin. Here the problem remained until revitalised by the mode of action studies initiated by Gale and his colleagues (1) in England and extended by Hotchkiss (14) in America.

Hotchkiss has emphasised the advantages of observing the action of penicillin on an enzyme preparation or on a washed cell system before it is confused by secondary processes, such as the reactions of normal growth, or secondary processes resulting from the distortion of normal metabolism. The available systems may be listed as follows. The first is growing cells continuously exposed to penicillin. This preparation contains dying, dead, and lysing cells. The second consists of growing cells briefly exposed to penicillin. This preparation, subsequently washed, contains penicillin-[illegible]own cells. The third is the original culture. Divided into two parts, one serves as a control, and to the other is added penicillin. Comparison between these two serves as the only means of showing differences directly caused by penicillin. Comparison between preparation one and the control shows differences between living and dead cells. Comparison between preparation two and the control shows differences between living and dying cells.

Whether penicillin would be capable of influencing the metabolism of washed cells must depend on the velocity of the reactions observed and of our ability to measure them. The experiments of Lester Smith *et al.* (8) appear to show that adequate amounts of penicillin are adsorbed to the resting cell interface.

While it is widely agreed that penicillin does not reduce the viability of resting bacteria, the reservation must be made that critical studies under the most favourable conditions have yet to be attempted. If absolute, it

merely means that an irreversible process can not be shown to occur. It remains to agree with Hotchkiss (14);

> Nevertheless, it is certainly reasonable to assume that if some part of the process of growth is vulnerable to penicillin, this partial growth process can eventually be studied in the proper kind of resting cell, or enzyme preparation without the occurrence of growth itself.

The effect of short exposures of penicillin on growing cells, that is, on dying cells, may now be discussed. In 1946 and 1947, Gale & Taylor (1, 15) reported that a particular strain of *S. aureus* Duncan grown for 4 hr. in a concentration of 10 units per ml. of penicillin, and then prepared as a washed cell suspension, differed 90 min. later from untreated cells in being incapable of concentrating glutamic acid from the external environment. This was the only way in which these cells could be shown to be different from normal cells. The interference with glutamic acid concentration could be observed also in cells during culture in penicillin-containing medium but if the bacteria were first washed, and penicillin then added, the cells did not differ from normal washed cells in their glutamic acid concentrating ability. It appears likely from this latter observation that the process of glutamic acid concentration was not itself damaged, but rather that some primary injury was caused by penicillin in growing cells, and this in turn led to interference with glutamic acid assimilation.

Similar evidence has been offered by Maass & Johnson (16) in support of this thesis. Gale & Rodwell (17, 18) later described a relation between penicillin sensitivity and dependence on preformed amino acids. Their experimental material was a collection of strains of *S. aureus* varying in penicillin resistance from 5.9 units per ml. to 6,000 units per ml. These were grown for 6 hr. and then washed well to eliminate utilization of glutamic acid. They were now treated with crystal violet at a final concentration of 1×10^6 to prevent diffusion of glutamic acid into the metabolic pool. They were now incubated for 1 hr. at 37° C. in a buffered medium containing 200 μl. of glutamic acid per ml., containing 1 per cent of glucose. It was found that the internal concentration of free glutamic acid was least in those cells most resistant to penicillin and greatest in those most sensitive. Cells resistant to 6,000 units per ml. of penicillin were Gram-negative and without any demonstrable glutamic acid. Thus, the ability of the cells to assimilate glutamic acid decreases as resistance increases.

The nutritional requirement of various strains was next studied and it was found that whereas the parent penicillin-sensitive cultures needed nicotinamide, aneurin, and a range of amino acids, the highly resistant strains could grow in a medium containing salts, ammonium ions, glucose, and aneurin. Mutants from these highly resistant strains showing a greater degree of penicillin sensitivity had also lost this synthetising ability. Finally, by growing the parent strains in serial sub-cultures in a medium from which essential amino acids were progressively withdrawn, they were trained to

synthetise their amino acid requirements, with the exception of cysteine and histidine, from ammonia and glucose. These nutritionally inexacting strains had a much higher resistance to penicillin than had the parent culture. More recently, Gale & Paine (19) compared the action of other inhibitors with penicillin, again using *S. aureus* Duncan, growth in a deficient medium and then exposed as a washed suspension. Of two of the processes studied, (*a*) accumulation of free glutamic acid, and (*b*) formation of cellular combined glutamate, sodium azide and 2,4-dinitrophenol are equally effective against both; 8-hydroxyquinoline is more effective against (*b*) than (*a*), while streptomycin affects (*b*) at concentrations higher than those needed to prevent growth. Under these conditions, as previously reported by Gale & Taylor (1), penicillin has no action. If, however, it is added during the period of growth, 30 to 60 min. before collection and washing, it inhibits both (*a*) and (*b*)

That penicillin might interfere with bacterial metabolism other than by prevention of assimilation of preformed amino acids was suggested by the experiments of Hunter & Baker (20). The organism observed was a strain of *Bacillus subtilis* which grows vigorously in a medium containing only ammonium sulphate as a source of nitrogen and does not release significant amounts of amino acids into the medium during growth. It produces no penicillinase. It is just as sensitive in the basal medium to the action of penicillin as it is in a complete tryptose phosphate medium.

Hotchkiss (22) was unable to repeat the experiments of Gale & Taylor (1) precisely. His strain was different (a bovine mastitis organism), his nutritional conditions different (a complete instead of a deficient basal medium), and the experimental conditions were different. It occasions no surprise that his results were different. Inhibition of glutamic acid was not more than 15 per cent, whereas in Gale's many experiments it is "complete."

Fortunately, the experimental effort yielded dividends in quite a different direction. Hotchkiss (21) had already discovered that in his experimental milieu washed staphylococci absorbed amino acids to form additional cellular protein to about one third of the initial value. In the presence of penicillin, washed cells do not increase in nitrogen but cause the disappearance of amino nitrogen at the same rate as in untreated cells. Analysis of the fluid outside the cells shows that nitrogeneous, non-amino substances accumulate there. Moreover, it is approximately equal to the amount of amino nitrogen, which in the absence of penicillin, would have been found inside washed cells in the form of protein. Penicillin inactivated by acid, alkali, or heating in neutral solution does not have this effect. The non-amino, nitrogenous material produced in the presence of penicillin gives, after concentrating, reactions characteristic of higher polypeptides. Estimates of the amount of peptide nitrogen corresponds to the amount of amino nitrogen disappearing from amino acids.

When Gale & Payne (19) examined washed suspensions of their strain of staphylococci (Duncan) in the presence of penicillin it had no capacity to cause an accumulation of glutamyl peptides in the external medium. But,

when they turned once again to the experimental conditions which had already revealed glutamic acid deviations in their hands, they were able to show accumulation of polypeptide. In view of the differences already described between the two strains, it appears significant that exposure during growth is again the requirement of Gale's strain. The essential requirement was to grow the organism for a limited period with penicillin. If a washed cell preparation is made within 30 to 60 min. after the addition (before the passage of glutamate into the cell has been completely inhibited), it is found that the cells no longer form combined glutamate. Free glutamic acid has disappeared, however, and up to 45 per cent of this can be recovered by acid hydrolysis from the supernatant.

It now seems possible to say that two bacterial cell preparations have been described in which the delicate injury caused by penicillin is ultimately indicated by a deranged protein synthesis.

Hotchkiss (22) believes penicillin to interfere with the bacterial synthesis of protein from amino acids, the process being blocked at a point which allows peptide to accumulate instead. The peptides may, of course, prove to be abnormal intermediates, or even to contain penicillin, or may prove to be degradation products of damaged bacterial protein.

Further evidence may be provided by a future study of the properties of the products produced when washed bacteria use amino acids in the presence of penicillin. Already a step forward has been taken in our understanding of the mode of action of penicillin and in addition a model has been designed for the study of bacterial protein synthesis.

Streptomycin

Streptomycin is an organic base which is active against many Gram-negative bacteria and mycobacteria. Authorities [cf. Henry & Hobby (23)] are agreed about its predominatingly bacteriostatic nature, although under suitable conditions bactericidal actions may be shown. Unlike penicillin, the activity of streptomycin is more readily influenced by external stimuli. For example, in an otherwise constant environment, the activity of streptomycin varies with the concentration, the size of the inoculum, the medium, and the pH. In this way it is like the sulphonamides. Streptomycin is most effective against dividing cells, but may have some effect against resting cells.

A grave disadvantage to the unrestricted use of streptomycin in chemotherapy is the frequency and rapidity with which resistant organisms emerge from initially sensitive strains. This is found also with test-tube experiments. In contrast, the emergence of resistant strains to penicillin is low. The streptomycin resistance ranges from cells only slightly more resistant than the original to absolute resistance [Demerec (24)]; with penicillin, the range is narrow.

At least three possible mechanisms of action of streptomycin have been described. The first is the reaction with certain desoxyribose nucleic acids described by Cohen (25). The precipitation, for such it is, between thymo-

nucleic acids and streptomycin and dihydrostreptomycin is caused by the diguanide component common to both [Ingrao & Vella (26)]. The fact that certain salts interfered with the precipitation of desoxyribose nucleic acid by streptomycin enabled Donovick, Bayan, Canales & Pansy (27) to conclude that the antibiotic mode of action was not due to this mechanism. A second possible mechanism of action is the inhibition of diamine oxidase reported by Zeller, Owen & Power (28). The third and perhaps the most important mechanism follows from the many early observations that streptomycin inhibited respiration in sensitive bacteria.

Perhaps the first systematic work of this kind on bacterial respiration was that of Geiger (29). If a strain of *Escherichia coli* was first allowed to oxidise fumarate, the subsequent oxidation of serine was much stimulated. The presence of streptomycin during fumarate oxidation prevented the stimulation. Umbreit (30) thought that streptomycin might be inhibiting the oxaloacetic-pyruvate condensation or analogous reactions, and employed a particular strain of *E. coli* to confirm Geiger's observations. The terminal respiratory processes of *E. coli* were next examined in a more direct fashion by Oginsky, Smith & Umbreit (31). The test organism was an aged suspension of *E. coli* kept in a refrigerator for a week to decrease its oxalocaetate decarboxylase content. The preparation oxidises pyruvate to the acetate stage, but oxaloacetate is but slowly oxidised. On the other hand, a mixture of oxaloacetate and pyruvate is oxidised rapidly. Without streptomycin, this oxidation goes to completion, whereas in the presence of 20 μg. of streptomycin per ml. the oxygen uptake is sufficient only for oxidation to acetate.

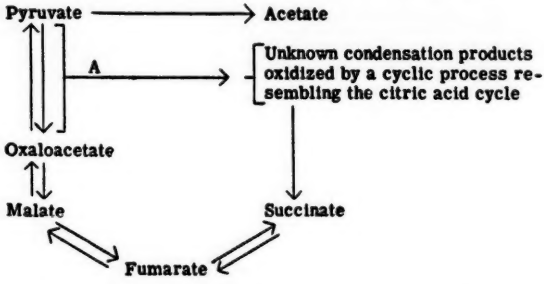

FIG. 2. Suggested terminal oxidation processes in *E. coli* [Oginsky, Smith & Umbreit (31)].

The interpretation favoured by the authors is that the antibiotic interferes with oxaloacetate-pyruvate condensation, rather than the alternative that the metabolism of "active acetate" is blocked. The antibacterial streptomycin derivatives possess this action; others do not. A 30 min. contact with the cells is necessary to make the effect irreversible. The suggested site of action of streptomycin on *E. coli* may be illustrated in Fig. 2. Reaction A is thought to be blocked.

The proof of the hypothesis would be the isolation of the responsible

enzyme and the demonstration of its inhibition with microgram amounts of streptomycin. This has not yet proved possible. In its absence, Umbreit (32) has listed five different reactions in which oxaloacetate is involved and which streptomycin modifies.

The first, the observation of Geiger (29), has already been discussed. He showed that the stimulation of amino acid oxidation by previous fumarate oxidation was probably due to oxaloacetate formed as an intermediate, which reacted in turn with the keto acid derived from the amino acid. The condensation of oxaloacetate with pyruvate derived from serine and α-ketobutyrate from threonine provided an explanation for the observed phenomena. Streptomycin inhibited the reaction.

The second was the observation that the technique of vacuum drying cells occasionally provided samples which failed to oxidise pyruvate. Umbreit (30) observed that the addition of fumarate or oxaloacetate to these cells promoted rapid oxidation which was inhibited by streptomycin.

The third was that although fresh cells of *E. coli* contained an active decarboxylase which rapidly converted oxaloacetate to pyruvate, Oginsky, Smith & Umbreit (31), found a marked difference in the effect of streptomycin on the two substrates.

The fourth noted the ability of ageing suspensions of *E. coli* to convert oxaloacetate to pyruvate to be gradually lost. However, they were able to oxidise a mixture of oxaloacetate and pyruvate, a reaction which is inhibited by streptomycin.

The fifth is that if streptomycin blocks the terminal reaction of pyruvate-oxaloacetate oxidation it ought to block similarly other substances, such as succinate and malate proceeding by the same route. This has proved to be so (31).

Is the reaction under discussion that which results in the death of the streptomycin-sensitive cell? All that can be said at this stage is that streptomycin, dihydrostreptomycin, and mannisidostreptomycin—antibiotics which inhibit growth—also inhibit this reaction. Inactive streptomycin derivatives do not.

Smith, Oginsky & Umbreit (33) made the interesting discovery that resistant cells do not have the oxaloacetate-pyruvate condensation enzyme. Nor indeed did dependant strains. It seems that strains growing in the presence of streptomycin eliminate the condensation. Its inexacting nutritional requirements, and its specialised metabolic pathways, appear to set *Mycobacterium tuberculosis* apart. No evidence of an oxaloacetate-pyruvate condensation has been uncovered, but an analogous mechanism involving the oxidation of fatty acids has been implicated by Umbreit (32).

Since it is known that enzymes responsible for oxaloacetate-pyruvate condensation in man are located in the mitochondria, it is of interest to know whether the reaction may be inhibited by streptomycin. When a cell-free mitochondria preparation in an enzymatically active state is exposed to streptomycin at 37°C., only high concentrations can inhibit. When, however, streptomycin is allowed to penetrate the mitochondria by contact

for 2 hr. in a refrigerator, Umbreit & Tonhazy (34) inhibited the oxidation of fumarate (and thus of oxaloacetate-pyruvate) by concentrations of 10 to 20 μg. per ml. of streptomycin. It may be that under certain conditions streptomycin inhibits oxaloacetate-pyruvate condensation in animal tissues as well as in the bacterial cell, but in the intact animal streptomycin does not usually penetrate the site of this reaction unless high intracellular concentrations are reached. Whether this is indeed the cause of damage to the eighth nerve in man remains to be investigated.

The Polymyxins

Five chemically distinct polymyxins, A, B, C, D, and E, have been described. All are basic peptides, the salts of which are water soluble, having a unique specificity for the Gram-negative bacteria. The known members have in common diaminobutyric acid, threonine, and a branched chain fatty acid, methyl octanoic acid, as molecular constituents. Polymyxin A contains leucine in addition, polymyxin B contains both leucine and phenylalanine in addition, and polymyxin C has phenylalanine in addition. Polymyxin D contains leucine and serine in addition to the common constituents and polymyxin E has the same constituents as polymyxin A, but has other distinctive features [Stansly & Brownlee (35)].

Reports of mode of action studies with polymyxins A and B by Brownlee and co-workers (36, 37) indicated that these antibiotics were bactericidal with extreme rapidity. They did not find that this action was much modified by environmental factors, although size of inoculum did modify it. They also believed it was unnecessary for the cells to be dividing to be affected. Similar effects were reported by Bliss, Chandler & Schoenbach (38) with polymyxin D, though they believed it to act only on the dividing cell. Hobby, Lenert & Dougherty (39) thought polymyxins A and B to be more effective against rapidly dividing cells, but to be bacteriostatic against cells which have failed to divide. All have commented on the low rate of emergence of resistant cells from otherwise sensitive strains.

The basic nature of these substances enable complexes to be formed with acids. The molecule which is formed is frequently insoluble. Many examples are known involving dyes, detergents, soaps, and salts. Antibacterial concentrations may be readily modified by these devices, but they are not thought to be concerned with mechanisms of action. As with streptomycin, lipositol also inhibits the action of the polymyxins.

No lead has yet emerged which throws light on the mode of action of these antibiotics.

Epilogue

"There is a Spanish proverb, which says very justly: Tell me whom you live with, and I will tell you who you are."

Philip Dormer Stanhope, Earl of Chesterfield.
Letter to his son. 9 October, 1747.

Let us finish where we began. The inability of microorganisms to grow in a given concentration of an antibiotic involves the selection of those

cells unable to survive in that concentration. It appears to have been possible to review the recent work on mode of action of antibiotics without discussing the problem of resistance. This is deliberate, since it does not appear necessary to our arguments to enter the battleground of the mutation versus the adaptation theory. The statistical method developed by Luria & Delbrück (40) which has provided much of the evidence for the mutation theory has been the subject of criticism, since variables other than mutation are included in the population. On the other hand, those who are critical of the adaptation theory point to the existence of strains resistant to concentrations of antibiotics greater than that in which they had been trained. [See, for example, the experiments of Silver and Kempe (41), Demerec (42), and Linz (43).] Those who are interested should consult a recent discussion by Barer (44) and for the present, the subject must still be considered to be under discussion.

The final understanding of the precise mode of action of antibiotics, non-toxic and toxic, will have immediate application to chemotherapy. Not less will be the wider applications which will follow the new understanding of bacterial metabolism incidently uncovered. The methods successfully developed for penicillin and streptomycin are now well understood. Only the techniques vary. The ripple which reaches the bank has an obvious origin in the stone thrown into the pool. Less obvious is the source of those ripples which finally beach in backwaters and creeks. For reasons like these the need to initiate and carry forward mode of action studies on the polypeptide antibiotics should be stressed.

From the point of view of mode of action studies, four groups of antibiotics fill the present scene.

Penicillin has been called an ideal chemotherapeutic agent. It is a weak acid, whose salts are soluble, are rapidly excreted, and are without toxicity. It has been studied most and we may soon expect to know something of its mode of action. Mycomycin, a new antimycobacterial substance, also a weak acid, is a potential candidate for this group.

A second, but basic group, also derived like penicillin from fungi, includes streptomycin and neomycin. These substances are toxic but useful. A beginning has been made with mode of action studies of streptomycin.

A third group comprises those extracted from bacteria. They are basic polypeptides of high molecular weight. Even when purified by exacting and meticulous physico-chemical tricks, they are usually mixtures of closely related substances. They are thermostable, diffuse slowly, adsorb readily, and are absorbed and excreted slowly. Some are quite toxic; some, like polymyxin E, are not. Included are the polymyxins, gramicidin, subtilin, circulin, bacitracin, and licheniformin. Nothing is known of their mode of action.

In a final group may be included antibiotics which show antirickettsial activity. Aureomycin and terramycin may prove to be similar chemically. Both are amphoteric substances reacting with acids and bases to form soluble salts. Chloramphenicol is a neutral substance. The mode of action of this group is unknown.

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PLANT CHEMOTHERAPY[1]

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Chemotherapy is both the newest and the oldest technique in plant pathology. From ancient times, "medicine men" of plant and animal pathology have drugged their patients to cure them of their maladies. Roach (66) cites a paper dated 1602 that recommends for "wormes" in trees a mixture of pepper, laurel, incense, and "good-wine." Today the number of research papers on chemotherapy of fungal, animal, and viral diseases of plants is on the increase, suggesting a renewed interest in the subject.

Concepts in Plant Chemotherapy

We must start with definitions, and we can begin at no better place than a definition of disease. We consider that disease is a "harmful deviation from normal functioning of physiological processes" (3), which usually results from continuous irritation by some agent called a pathogen. It is impossible to ascertain exactly when this harmful deviation from normal processes begins. For reasons of exactness of definition, it will be assumed that the fungus can cause disease from the moment it enters the host. In this paper we shall deal with all types of pathogens: fungi, bacteria, viruses, nematodes, arachnids, and insects, but shall arbitrarily exclude deficiency diseases.

In defining chemotherapy, the chief problem is to distinguish the concept of chemical protection from that of chemical therapy. If the pathogen is killed before it can enter and produce disease, the plant has received chemical protection. If the pathogen is killed after it has entered and produced disease, however slight, the plant has received chemical therapy. In the first instance, the disease has been prevented; in the second it has been cured. In the first instance, the pathogen is wholly outside the plant; in the second it is wholly or at least partially inside the plant.

It follows from the position of the pathogen in the two cases that the compound must be outside the plant to protect it, inside the plant to effect therapy. Time of application of the compound is not critical, where and when it acts is.

Under special conditions, a compound inside can provide protection. To do so, it must diffuse out into the infection drop and kill the pathogen before it begins to invade the host. This has not been accomplished experimentally, as far as we know, but protocatechuic acid and catechol act in just this way in onions resistant to smudge, according to Walker (92).

Similarly, under special conditions a compound outside can diffuse inward and accomplish therapy. This has been achieved experimentally and even commercially in several cases, such as phenyl mercury compounds for apple scab and internal cereal smuts.

[1] The survey of literature pertaining to this review was concluded in February, 1951.

In addition to the killing or inhibition of pathogens, chemicals may antidote the toxins produced. If they do this, they are chemotherapeutants.

Perhaps we can now set down a workable definition of chemotherapy. Chemotherapy is treatment of the host with a compound so that the action of the compound occurs inside the host when the host is diseased. It may (*a*) kill the pathogen as it enters the host, (*b*) rid the host of an established pathogen, or (*c*) mitigate disease. Howard & Horsfall (54) have discussed concepts in chemotherapy.

Chemotherapy is not limited to systemic action. It includes topical action as well. A chemotherapeutant may act at the site of application or at a distance from it. If the compound is applied to the infection court, it is topical. If it can be applied elsewhere and translocated to the site of infection, it is systemic.

The treatment of crown gall with penicillin by surface painting or injection is an example of topical action, even though the compound may diffuse slightly into the gall (10). The treatment of a leaf spot by soaking a nearby leaf in the compound is an example of systemic action even though the leaf spot is a localized lesion.

Topical Chemotherapy of Local Diseases

Some of the most successful work in plant chemotherapy has been done with local diseases, galls for example. As early as 1891, Bolley (5) showed that potato scab, a tumor on the tuber, could be cured by soaking the diseased potato in a mercuric chloride solution. Fifty years later, Strong & Cation (84) cured the rust gall on cedar with sodium dinitro-*o*-cresylate, and chemotherapy of galls began to roll. Positive action on bacterial crown gall by topical applications has been reported by Ark (4) with sodium dinitro-*o*-cresylate, by Brown & Boyle (9, 10, 11) with penicillin, and by Hampton (44) with penicillin and streptomycin. DeRopp (21) concluded that streptomycin exerted its effect on the bacteria, penicillin on both the tumor and the bacteria (22). DeRopp's latest paper (23) shows that several folic acid analogues that are effective on animal cancers are also effective against crown galls on carrot tissue in culture.

Topical chemotherapy of surface lesions like apple scab would seem an obvious possibility, but it has received serious attention for less than two decades.

In 1931, Hamilton (42) cured scab with lime sulfur and organic mercury (43) early in the disease year, and his theory went on to commercial adoption.

Yarwood has investigated chemotherapy of rust lesions (95). He showed that topical application of copper sulfate would cure bean rust if applied fairly shortly after inoculation. Later (96) he showed that hydrogen sulfide and hydrogen cyanide gases are more effective, presumably because they are more penetrating. His most recent paper shows that radiosulfur accumulates selectively in the lesions (97).

Small cankers on dormant stems can be combated with fairly caustic therapeutants. Viala (91) opened this field in the nineties by spraying an-

thracnose cankers on grape canes with copper sulfate. Forty years later, Truffaut & Pastac (87) proposed that sodium dinitro-*o*-cresylate be used on dormant stems. Wilson (94) cured *Sclerotinia* cankers on apricot twigs with calcium arsenite.

Hiltner (48) was first to recognize that deep infections in seeds could be cured by application through the uninjured surface. He cured rye seeds infected with *Fusarium* by soaking them in mercuric chloride. Organic mercuries soon replaced mercuric chloride and thousands of bushels of cereal seeds infected with *Fusarium*, *Helminthosporium*, and *Ustilago* are treated today in many parts of the world.

In 1940, Nugent & Cook (63) succeeded in curing rape seed infected with bacterial black rot by fumigating the seed with chloropicrin. This work should be extended, since it looks interesting.

Systemic Chemotherapy of Local Diseases

Systemic chemotherapy for a local disease, as one might expect, is the oldest technique of all [see Roach (66)].

Fungal pathogens.—Copper sulfate and the salts of heavy metals always command a share of attention when fungal infections are under consideration. Kinney (55) noted that potato tubers were freer of scab on plants sprayed with bordeaux mixture than tubers on unsprayed plants, presumably because tubers on sprayed vines are higher in copper content than those on unsprayed vines (58).

In 1930, Horsfall (49) suggested that tomato seedlings probably absorbed enough copper from a seed treatment to make them resistant to attack from soil organisms at a distance from the seed. Bouchereau & Atkins (7) have shown that rice seedlings do absorb enough copper from seed treatment to become resistant to *Helminthosporium*.

There are reports that the following foliage diseases may be controlled by feeding copper sulfate to the roots of plants; rice blast (1), downy mildew of lettuce (32), and powdery mildew of barley (64).

A few isolated reports have been noted on the use of other inorganic salts. Borax fed to wheat plants reduced leaf rust and stripe rust [Gigante (40)], and when fed to flax reduced rust [Heggeness (47)]. Sodium thiosulfate injected into apple reduced powdery mildew (Roach (66)], and metallic sulfates reduced crown gall on castor bean [Verona (90)].

Organic therapeutants flared into sudden prominence with Domagk's (31) discovery of Prontosil in 1935 for human therapy. Gassner & Hassebrauk (39) shortly thereafter reported that sodium toluene-sulfonamide reduced wheat rust when it was fed to artificially-inoculated plants in the greenhouse. Out of 174 organic compounds tested they reported some success also with picric acid, acridine, and one-bromo-2-naphthol. Their work on sulfonamide was confirmed in the greenhouse by Hart & Allison (45), but Hassebrauk (46) was unable to confirm his results in field experiments. This is a curious hiatus that needs attention.

Caffeine and theobromine increase the resistance of beans to rust, but

have no effect on wheat rust, according to Sempio (71).

By injection of thymol into pear shoots, Scherer (70) obtained some reduction in bacterial fire blight, but none was obtained by Rudolph (68) when he injected penicillin into pear for fire blight or walnut for bacterial blight.

Aryloxyaliphatic acids are reported by Crowdy & Wain (17) as systemic chemotherapeutants. They fed the test compounds to broad beans (*Vicia faba*) in water culture, and then inoculated the foliage with *Botrytis fabae*. 2,4-6-Trichlorophenoxyacetic acid seemed best. ". . . the effect of the treatment was to check the spread of the individual lesions rather than to prevent lesion formation." Nevertheless, the effect was demonstrably chemotherapeutic. Stewart (74) reported that spraying citrus trees with 2,4-dichlorophenoxyacetic acid or 2,4,5-trichlorophenoxyacetic acid during the three months preceding harvest reduced the incidence of *Alternaria* (*citri*) decay on the stored fruit. The latter was the more effective compound.

McNew & Sundholm (61) immersed one leaf on each tomato plant in suspensions of several 1(substituted phenyl)-3,5-dimethyl-4-nitrosopyrazoles for 36 hr. The treated leaf was severed and the other leaves were inoculated with *Alternaria solani*. They obtained appreciable reduction of disease on the untreated leaves. Clearly, the compound had been absorbed through one leaf and translocated to the other leaves.

One of the most interesting instances of chemotherapy for local lesions is the fumigation of tobacco seedlings with benzene for controlling downy mildew [Angell, Hill & Allan (2)].

Animal pathogens.—The attack of various animals on plants may be considered in the light of local invasion. DeLong *et al.* (20) fed copper sulfate to bean plants in water culture and killed leafhoppers on the leaves. Similarly, Fulton & Mason (37) showed that rotenone could be translocated in plants. Little was made of the results at the time, but interest in this field also has increased recently.

Martin (59) of England participated in the scientific survey of Germany after the war. He learned that Schrader and Kükenthal had found that *bis* (bisdimethylaminophosphonous) anhydride and other compounds possessed chemotherapeutic qualities against insect pests. According to Ripper *et al.* (67), Martin suggested research on the subject, and this is reported *in extenso* in a recent paper (67). Suffice it to say that the compound can kill aphids on leaves whether it be applied to foliage, to soil in which the plant is growing, or to cut stems. David (19) reported results with the compound made from radioactive phosphorus 32. He found that only a few hours are required for the compound to be absorbed from the soil or through the foliage and distributed throughout the plant. Soil reduces the uptake of the compound more than sand. Aphids fed on radioactive plants became radioactive and secreted radioactive honey dew. Since honey dew contains sugar, David's results suggest that some radioactive glucose phosphate was excreted.

Parathion is chemically related to the phosphonous anhydride discussed above. Questel & Cronnin (65) showed in 1947 that if parathion were watered on corn plants, borers in the stem would be killed. Grainger (41) fed parathion to potatoes and killed aphids on the foliage. Tarjan (86) reported simi-

lar results for root nematodes on *Buxus* (box). Starnes (73) stated that an extract of potato foliage grown on soil containing benzene hexachloride was toxic to mosquito larvae.

Sodium selenate has been used as a systemic compound for control of red spiders on roses. It received commercial acclaim for a short time. Rose growers watered their plants with it. It soon began to fail, however, because the red spiders developed a resistance to it. Dimock (24) was able to cure chrysanthemums of leaf nematodes by watering them with sodium selenate.

Systemic Chemotherapy of Systemic Diseases

Systemic diseases beckon powerfully to the explorer of chemotherapy. They are beyond attack by protective sprays. A systemic disease like a systemic chemotherapeutant is one in which the pathogen itself or its toxic by-products move in the conducting system of the plant away from the infection court.

Vascular diseases.—Rumbold (69) gave the theory of chemotherapy a good trial on chestnut blight in the second decade of the century. Her results were mostly negative, but she did suggest a possibility that developed later, namely, that the toxin could possibly be antidoted chemically. Howard (53) made good on this proposal. With his colleague Caroselli he showed (12) that bleeding canker of hardwoods is a toxin-induced disease which could be mitigated, if not cured, by antidoting the toxin with injections of diaminoazobenzene dihydrochloride. Later Feldman joined the team which has proposed recently (34) a mixture (Carolate) of calcium hydroxide, urea, salicylate, and "an azo dye" for the control of Dutch elm disease by soil injection. Cecconi (13) earlier recommended lime for Dutch elm disease.

The work in the laboratory at The Connecticut Agricultural Experiment Station has centered on the X-disease of peach, a virus, and on the Dutch elm disease, a vascular wilt disease. By 1940, we could see that the elm disease could never be eradicated. Having been mildly interested in chemotherapy for 10 years, we began to attack elm disease that way. 8-Quinolinol sulfate was selected from a series of water-soluble fungicides under investigation and a brief report on its action to alleviate symptoms was published in 1941 by Horsfall & Zentmyer (51, 52). A later report was published by Zentmyer *et al.* (98). Literature search disclosed some earlier work on the substance as a chemotherapeutant for vascular diseases, but the results were discordant [Boudru (6), Garbers (38), Brooks & Storey (8), Fron (36)]. Fron finally claimed positive reduction of Dutch elm disease and of *Fusarium* wilt of carnations with this compound.

Researches of Dimond (25, 29) and Stoddard (79) have revealed some of the reasons for discordant results: (*a*) the effects of 8-quinolinol sulfate are evanescent (98), whereas 8-quinolinol benzoate is more persistent. (*b*) Since the effectiveness is essentially nil on established infections, the compound must be in the tree when infection occurs (25, 29); it is enjoying commercial usage of that type. (*c*) Some workers injected the compound; this is the least effective method of administration because of distribution troubles. (*d*) Fron (36) watered the compound onto the soil; this improves the distribution

and with it the results as compared with injection. (*e*) The best method of treatment is to inject it into the soil around the feeding roots (26, 29).

The answer to Dutch elm disease is not yet in hand. 8-Quinolinol benzoate and Carolate are much better than nothing, but they are still only palliative.

Fusarium wilt of tomato and carnation have also received attention [Dimond *et al.* (27), Stoddard & Dimond (83)]. The following chemotherapeutants have shown good to excellent control of one or the other of these: 4-chloro-3,5-dimethyl phenoxy ethanol, 2-norcamphane methanol, 8-quinolinol sulfate, n-octadecyl trimethyl ammonium pentachlorophenate, and N-(4-nitrophenyl)-3,4-dichlorobenzene sulfonamide.

Systemic bacterial blight of garden bean has responded to some extent to chemotherapy with salicylic acid and auramine (30).

Virus diseases.—Chemotherapy of virus diseases was forecast in an interestingly prophetic paper on peach yellows in 1845 by Darling (18), a lawyer and farmer of New Haven, Conn. Darling conjectured that peach yellows is caused by a poison (now called virus), coming from an insect, and that it should be possible to combat the poison "if diseased peach trees should be made to absorb a variety of matters . . . that will . . . decompose the poison."

It is of interest to note here that Stoddard, a plant pathologist also of New Haven, reported almost 100 years later that the "poison" of X-disease of peach could be decomposed (i.e., inactivated) in living peach buds by "a variety of matters," including quinhydrone, hydroquinone, 8-quinolinol sulfate, *p*-nitrophenol (75).

Continuing this work, Stoddard (76) reported that small trees could be protected from X-disease by injecting them through the cut upper end of the main stem. The most promising treatment was with *p*-aminobenzenesulfonamide. Its effect was antidoted by *p*-aminobenzoic acid as in the case of bactericidal activity. Substituting a methyl group for the amino group reduced the activity. A summary of the research on chemotherapy of X-disease of peach has been published by Stoddard (77).

Limasset *et al.* (56) and Locke (57) simultaneously reported in 1948 that 2,4-D derivatives have an effect on viruses. Locke used 2,4-dichlorophenoxyacetic acid for treating leaf roll of potato. Limasset *et al.* used 2-methyl-4-chlorophenoxyacetic acid on potato virus X and Y on tobacco. Both papers reported that the effect eventually wore off. Presumably the virus was inhibited from multiplying at first; symptoms disappeared and later returned.

Takahashi (85) has shown and Stoddard & Dimond (80) have confirmed that malachite green can delay reproduction of tobacco mosaic in the host.

Effect of the Therapeutant on the Pathogen

A successful chemotherapeutant may participate in any or all of three systems: the host, the pathogen, and the toxin. In so doing it may in turn be acted upon by them.

Most of the successful chemotherapeutants listed in the preceding pages are toxic to the pathogens concerned. These include lime sulfur, copper sulfate, penicillin, 2,4-6-trichlorophenoxy acetic acid, nitrosopyrazole, 8-quin-

olinol, *bis* (*bis* dimethylaminophosphonous) anhydride, parathion, rotenone, etc. Undoubtedly, many new chemotherapeutants will be discovered that are toxic to various pathogens.

It would be very easy to be misled by this simple generalization. Toxicity to spores bears little or no relation to chemotherapeutic activity against *Fusarium* wilt of tomatoes (27). This may be so because the host activates some compounds and inactivates others. The host appears to activate the *bis* (*bis* dimethylaminophosphonous) anhydride (67) for aphids, copper sulfate for powdery mildew (95) and 4-chloro-3,5-dimethylphenoxyethanol for *Fusarium* wilt (27, 78).

Effect of the Therapeutant on the Fungus Toxin

Another method of evaluating chemotherapeutants is based upon the conception that some pathogens injure the host primarily through the toxic compounds that they excrete [Howard (53)]. In brief, the procedure consists of growing the fungus in a nutrient solution, and mixing the test compound with the fermented nutrient. Tomato cuttings are placed in the test solutions. If the test compound reduces the wilting action of the toxins, the compound is regarded as a promising chemotherapeutant [Feldman *et al.* (35)]. This conception of chemotherapeutic action is more limited in scope than the one discussed above, but has resulted in promising compounds.

The method of antidoting toxins is rapid and less empirical than the direct assay, but it will select no compounds whose activity has a different basis. Other difficulties may arise, viz: (*a*) Neutralizing the toxins which a pathogen produces does not prevent it from forming more of them [Dimond *et al.* (29)]. Since the therapeutant is used up in the process, the disease can be alleviated but seldom cured through this approach. In contrast, if the pathogen is eliminated, toxin formation ceases. (*b*) The toxin in culture may not be the same as that formed in the diseased plant. If this is true, results based on toxins from culture may be artifacts (29). The conditions under which *Ceratostomella ulmi* forms phytotoxic components in its medium, are highly specific, being governed by variations in pH and source of nitrogen [Feldman *et al.* (35)]. Even in the host, the formation of toxins by this fungus is highly specific (29). One may question whether a phytotoxic fraction produced *in vitro* is necessarily the equivalent of a toxin produced by the pathogen in the host. This approach may advance most surely when one knows with what specific toxin he has to deal. (*c*) Analysis of the phytotoxic material in media on which *Ceratostomella ulmi* has grown reveals that not one, but several toxic components are present (29). If some of these are artifacts (i.e., if they are not produced in the diseased elm), the criticism just presented is quite important. If all of these toxins are produced in the sick plant, then not one but several chemotherapeutants may be necessary to negate the effect of the fungus. Problems of distribution become highly complex under these conditions.

When it happens that the toxic compounds which the pathogen excretes are essential for its continued growth, e.g., an extracellular enzyme, then the method will select compounds which reduce the growth of the pathogen or eliminate it.

Effect of the Therapeutant on the Host

A first principle in chemotherapy is that a compound must be low in toxicity to the host (tolerated dose) while being effective in mitigating disease (effective dose). The tolerated dose of topical therapeutant must be learned while the test is under way. A compound may be essentially bland to an uninjured cuticle but very toxic if the cuticle is ruptured.

The tolerated dose of a systemic therapeutant may be tested with cut shoots or by standing the plant roots in test solutions. In most cases, a cut shoot will be injured more than an intact plant if it absorbs solution from the basal end, but this will differ with different compounds.

Direct Assay of Therapeutants on the Host

The direct measurement of chemotherapeutic activity has the advantage that it approximates the conditions under which the compound must ultimately perform, and results based upon it may need fewer corrections than those based upon experiments with toxins only. Since no underlying assumptions are made as to mode of operation in the plant, compounds which are effective by any or all mechanisms are selected. However, the procedure is most tediously slow. Such an approach has been carried forward extensively in this laboratory with Dutch elm disease [Zentmyer *et al.* (98)] and with tomato wilt. In these experiments we have had most success with *Fusarium* wilt of tomato. Tomato plants are grown in sand and are so fertilized with Hoagland's solution as to be highly susceptible to *Fusarium* wilt [Walker & Foster (93), Stoddard & Dimond (81)]. When the plants are in the four leaf stage, compounds in aqueous solution are applied at the tolerated dose to the sand in which plants are growing. After a suitable period of treatment (usually 10 days), the plants are uprooted and washed under running tap water to remove the sand and test compound from the root surface. The roots are then wounded extensively, since *Fusarium lycopersici* enters through wounds. The wounded roots are dipped momentarily in a heavy suspension of bud cells of the pathogen. Then the plant is repotted in clean sand in a fresh pot and held for further observation. After a suitable period, the severity of disease is compared with that of an untreated, inoculated plant. Under these conditions, the compound under test is present primarily within the plant, and control of disease, if any, is a result of its ability to kill the pathogen as it enters [Chapman (15)], Horsfall *et al.* (50), Dimond *et al.* (27)]. Using the same principles, Crowdy & Wain have used *B. fabae* on leaves of horse bean to measure activity of systemic fungicides (17).

Routes of administration for adequate uptake of compounds.—Compounds have been applied to roots, stems, and leaves for chemotherapeutic purposes. Stem injection was used in the earliest studies of this subject (60, 62, 66). Absorption through leaf surfaces has been employed more recently (61, 66). Compounds have also been applied to sand or soil in which plants are growing and then absorption of the compound may occur through roots (29, 77, 79) (see also topical references).

Except in rather special circumstances, the techniques of introducing compounds into the stem (60, 66) seem to offer little promise in the chemo-

therapy of plant diseases [see Brown, however (9, 10, 11)]. Stem tissues are well adapted for longitudinal transport of materials in solution, but are very poorly adapted for radial and tangential movement (29, 82). As a result, when solutions are injected into the stem they are not evenly distributed in the tissue. When 8-quinolinol benzoate was applied to roots for chemotherapy of Dutch elm disease, the results were more uniform, and the effect was much more significant than when it was injected into stems (29). Similar results were obtained when beans were treated for bacterial blight (27), and when peaches were treated for X-disease [Stoddard (77)]. In the latter case, solutions were introduced into the terminal end of cut stems of peach seedlings as compared with solutions of the same compound applied to roots (76, 77). Roach has devised methods of injecting solutions into trunks of trees so that more or less uniform distribution is obtained. However, these techniques involve considerable experience, some study of the individual plant to be treated, and more labor per plant than could be expended except where individual plants have high value.

Some work has been done with absorption of the compounds through the leaf surface. Roach has shown that when individual leaves are immersed in solutions, the compounds are absorbed and become distributed in the plant in accordance with its trace anatomy (66). To some extent the successful use of leaf application of compounds will depend on the ability of the compound itself to enter the leaf surface. The absorption of 2,4-dichlorophenoxyacetic acid by leaves is an outstanding case of this ability. Clayton's work on the ability of fatty acids to control downy mildew of tobacco (16) may be a result of their absorption into the leaf.

Distribution in the plant.—When a compound is absorbed into the plant, its distribution may or may not be systemic. In the case of the systemic insecticides, there seems little doubt that, when absorbed by the root, these compounds are freely translocated (59, 67, 88). Evidence of systemic distribution of 8-quinolinol benzoate in elms when applied to their roots has already been noted (29). More recently, Crowdy & Wain have used compounds which combine the translocatability of the aryloxyaliphatic acids with fungitoxicity and have demonstrated, by virtue of their chemotherapeutic effect on horse bean inoculated with *B. fabae*, the fact that they become systemic in the plant (17).

Systemic distribution is not necessarily desired in all cases of chemotherapy. One phase of chemotherapy is concerned with killing the pathogen as it enters the host, and a pathogen usually enters the host at a characteristic locus. Since *Fusarium* and *Verticillium* are soil borne, they usually enter the host through roots and may invade stem tissue only after they become established. *Ceratostomella ulmi*, being carried by *Scolytus* bark beetles, usually is deposited in twig wounds in the elm by its vector. Bacterial blight of beans usually enters a leaf through stomata. If these organisms are to be combated by chemotherapeutic means as they enter the plant, it is necessary only that the compound be concentrated in the host at the locus of infection.

Evidence for this point of view is not lacking. N-(4-nitrophenyl)-3,4-dichlorobenzene sulfonamide is an effective chemotherapeutant against *Fu-*

sarium wilt on tomatoes, and has some toxicity to mycelium of *F. lycopersici.* Yet when placed in high concentration on filter paper pads which lie on potato dextrose agar, heavily seeded with *F. lycopersici*, it produces no zone of inhibition. Evidently it is adsorbed on the negatively charged cellulose fiber of the filter paper (14, 27). In the plant, too, it presumably would behave similarly, and if taken into root tissue would be absorbed there and not be translocated. Yet this would be the very distribution which would most effectively weaken the pathogen as it entered the root.

We regard the systemic fungicide, therefore, as but one type of chemotherapeutant, and the compound which remains fixed in a tissue by being absorbed as another. Each will be useful in treating certain types of diseases. The factors which govern the chemotherapeutant to be used in a specific case, under this view, include the disease being combated and its characteristic locus of infection, the nature of the tissues at this locus, the site of application of treatment, and the properties of the compound to be used, with special reference to translocatability and substantivity.

Stability of chemotherapeutant in the plant.—We have already emphasized that compounds may be introduced into the plant prior to inoculation in chemotherapeutic use. When this is done, the value of treatment is in part measured by the period of time over which a pathogen entering the plant will be killed or weakened.

A compound which is biologically inert is unlikely to act as a chemotherapeutant. Compounds which are active may vary considerably in their stability when in contact with host tissues, but one may expect that the longer they are in the host, the less effective they will be. This may arise from two causes: (*a*) slow breakdown of the compound in the host, and (*b*) the effects of growth of the plant to dilute the chemotherapeutants at the locus of infection. The second effect will operate differently, depending upon whether a compound is systemic or substantive. If systemic in distribution, the chemotherapeutant may be diluted below the effective dose by growth of the host; if substantive, it will be fixed in tissues so that subsequent growth will be unprotected. Dimond *et al.* (27) have measured this effect with chemotherapeutants for *Fusarium* wilt of tomato. The result of these effects makes necessary serial application of all but the most effective compounds.[2]

Stability of chemotherapeutant in the soil.—We may expect many chemotherapeutants to be broken down in the soil in a reasonably short time. If this breakdown is too rapid, the compound becomes worthless. For example, tomato plants were grown in soil and in sand, and treated each with N-(4-nitrophenyl)-3,4-dichlorobenzene sulfonamide as a chemotherapeutant for *Fusarium* wilt. Treated plants grown in sand showed very slight amounts of *Fusarium* wilt, whereas comparable untreated plants were heavily diseased. In contrast, treated plants grown in soil were almost as heavily diseased as plants in soil which received no treatment (27).

On the other side of the picture there are compounds which break down

[2] The systemic insecticides appear at present to be uniquely stable in the plant. It is also apparent that the amount which can be present in the plant without injuring it is much greater than the effective dose for controlling insects.

very slowly indeed in the soil, among which are benzene hexachloride and DDT (72, 89). Such compounds, if chemotherapeutic, may be useful if they are so used as not to accumulate in soils with unfortunate side effects.

Between these extremes will fall many compounds whose stability in soils is sufficient so that a plant may absorb them and be benefitted by their chemotherapeutic effects, but which do not persist in the soil [Stoddard & Dimond (83)].

Timing of treatments.—Evidently serial application of chemotherapeutants will be necessary in practice. A number of factors may govern the frequency with which applications must be made. Some of these may be noted.

(*a*) Breakdown in the soil reduces what is available for absorption by the plant. Breakdown of chemotherapeutant in the plant reduces the dosage below what is effective for disease control. Each of these trends creates a problem of maintaining the effective dose, and each must be counteracted by supplying more material serially to compensate for what is lost by decomposition. When reasonably stable compounds are used, it may be unnecessary to make such frequent successive applications, as, for example, in the case of the systemic insecticides.

(*b*) Plants may vary in their period of susceptibility to diseases. When the object of treatment is to kill the pathogen as it enters, the plant must be supplied with an effective dose during the entire period that it is susceptible. In the case of *Fusarium* wilt of carnation, where the plant is more or less susceptible during its entire life, Stoddard & Dimond applied 4-chloro-3,5-dimethyl phenoxy ethanol in a commercial greenhouse, and reduced the number of infected plants by half when applications were made each week (83).

The case of Dutch elm disease is of interest here. Dimond *et al.* (29) have noted that when 8-quinolinol benzoate is absorbed by healthy elms and kills the pathogen as it invades the host, treatment is necessary only annually. What governs frequency of application in this case is the period of high susceptibility of the tree to disease and the habits of the beetle which is a vector. On the basis of studies of the carbohydrate levels in elms, Feldman *et al.* (33, 35) concluded that elms are most susceptible to Dutch elm disease when the carbohydrate levels in the tree are at a minimum. This was found to occur in the spring at approximately the time when the peak of the spring emergence of bark beetle (which is the vector) occurs. Thus it happens that maximum susceptibility coincides with maximum inoculation of trees. At other times of the year, the fungus is carried to the elm by a second brood of beetles, but its chances of invading the tree are low.

Relation of dosage to amount of pathogen to be overcome.—There must be a relation between the quantity of pathogen present in the plant and the quantity of chemotherapeutant which will be required to overcome it. Such a relation is readily measured in spore germination tests and is known to be a direct proportion in the protective fungicide field (28). At present there is no easy way of making such measurements directly with chemotherapeutants and one must rely on indirect evidence which merely suggests a comparable relation.

Stoddard, in his studies on the X-disease of peach, has reported greatest

success from treatment with compounds generally when applications were made after the diseased bud was placed in the healthy stock (76, 77). In sharp contrast, Dimond *et al.* report that in the case of *Fusarium* wilt of tomatoes and bacterial blight of beans, success is closely dependent upon the timing of treatments before inoculation occurs, and inoculation must closely follow the last application of chemotherapeutant (27). One may relate this behavior to the growth of the pathogen in its host. The longer the period which elapses between entry of the pathogen into the host and the commencement of treatment, the greater the mass of pathogen to be overcome by the chemotherapeutant. When considered in this light, one may account for the apparent discrepancies between Stoddard's observation on X-disease and those of Dimond *et al.* on *Fusarium* and bacteria in plants. To transmit X-disease to a peach experimentally it is necessary to insert a diseased bud or piece of bark into healthy tissue. The virus probably does not multiply appreciably for a period following this operation, and translocation across the grafted zone is probably exceedingly limited. If, during this phase, the chemotherapeutant is already in the plant and no more is applied, it is probably well degraded through enzyme action or through translocation before it can act upon the virus at the point it enters the healthy tissue. It probably cannot be translocated in quantity into the diseased tissue until a few days have elapsed during which time the grafted tissues become united and grown together. If chemotherapeutant is supplied during the phase that this process is going on, then materials enter the infected tissue and may inactivate the virus while it is poorly able to withstand such treatment.

Similarly, if chemotherapeutant is already well and freshly supplied to tomato roots and *Fusarium* spores are then placed on the wounded roots, these spores will encounter fresh chemotherapeutant and be hindered in their invasion of tissue. If, on the other hand, spores are applied to wounded roots very long after chemotherapeutant has been applied, a sufficient breakdown of chemotherapeutant may have occurred so that the fungus is only slightly inhibited in becoming established in the host. If inoculum is applied well before chemotherapeutant, it is already so firmly entrenched in the host that the dosage of compound which may be accumulated by a given amount of tissue is less than is required to inhibit the fungus.

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PATHOLOGICAL PLANT GROWTH[1]

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Introduction

The changes from normal growth to diseased growth involve many fundamental biological problems. While we are concerned here primarily with the diseased growth in plants, the problem is basically one concerning all kinds of living cells. The more we learn, the greater appear the metabolic parallels between "cabbages and kings." From the standpoints of growth stimulation or inhibition, many basic substances, including various carbohydrates, fats, proteins and their derivatives, mineral salts, vitamins, and enzymes are common factors occurring both in plant and animal cells.

For fundamental work on growth, plants have certain advantages. For example: (*a*) Plants have no complex nervous, digestive, and circulatory systems which complicate the basic physiology. (*b*) Large numbers of plants may be used at a relatively low cost. For the most part, we determine the statistical significance desired and use the number of plants necessary to achieve it. (*c*) Plants permit many and diverse experimental manipulations, as discussed later. (*d*) The possibilities for genetic purity with plant material are real and important. Many inbred lines are available for use. Still better, many kinds of plants, such as potatoes and many fruits, are ordinarily reproduced by vegetative propagation. Thus, the different individuals are genetically identical. (*e*) For details of tissue metabolism, tissue cultures with plant material offer a relatively simple and direct approach not yet possible with tissue from higher animals. These cultures grow upon media which contain only nutrients with known chemical formulas. Ordinarily the tissues grow well without a change of nutrient for some weeks. The growing tissue develops in a compact mass easily separated from the medium. Thus, any change in growth may be determined merely by weighing the tissue pieces. (*f*) Many ways are available for inducing at will one or another kind of diseased growth.

Causal Agents for Diseased Growth

A number of physical, chemical and biological agents can induce pathological growth. Among them are the following:

Physical agents.—An excess of vapor tension often stimulates considerable cell enlargement, particularly from the lenticels of certain woody

[1] The survey of literature pertaining to this review was concluded in December, 1950.

[2] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The writers gratefully acknowledge suggestions from D. R. Goddard, and R. S. DeRopp.

plants (76). Wound overgrowths frequently develop where the downward passage of elaborated food has been obstructed (77). This is commonly found where a graft union is inadequate either mechanically or physiologically (69). Diseased growth considerably beyond that necessary for healing occurs in some plants following injury.

Chemical agents.—A large number of chemicals are capable of inducing more or less diseased growth. Smith (83) began this work which has been extended widely by many other workers since then. A compilation of such chemicals was listed by Riker & Berge (72). The phenomenon is so common that one can expect diseased growth to follow the application of almost any chemical at a concentration which induces injury but does not kill. In some cases only a little growth appears in addition to a healing response. In others, as with many growth substances, conspicuous galls develop, as considered later.

Genetic constitution.—Many plants seem to carry, as a portion of their genetic makeup, the capacity for forming diseased growth of one kind or another. Among these may be included the burls, well known on various trees; the nonparasitic burr knots on many apple trees (85); and the galls on a number of tobacco hybrids, for example, *Nicotiana glauca* Grah., ♀ by *N. langsdorffii* Weinm. ♂ (45, 48).

A wound tumor virus.—A virus induces galls on many infected legumes (2). Wounds are involved in the origins of at least many of the tumors.

Bacteria.—A number of bacteria induce diseased growth on various plants. These have been compared by Riker, Spoerl & Gutsche (80). Among these the crown gall bacterium, considered later, has received much more study than the others.

Fungi.—Many fungi are capable of inducing diseased growth of various kinds. Among these may be included such well-known diseases as cabbage club root, cedar-apple rust galls on red cedar, the witches-broom on hackberry, the smut galls on corn, and parasitic burr knots on apple (10).

Nematodes.—A number of pathogenic nematodes induce galls on their host plants. One of the best known is the nematode gall on the roots of tomato (27).

Insects.—An enormous number of insects stimulate galls of a great many different kinds on their respective host plants. These have been described by Felt (21) and Felt & Rankin (22).

The living agents, through their metabolism, induce both physical and chemical changes, commonly at a low level but for long periods. This compares with hormones applied to the outside of plants. The more effective chemicals are commonly applied in fatty carriers. Thus they also occur at low levels for days and weeks.

What actually initiates these various diseased growths, what keeps them going, and how they can be inhibited are critical questions. They have stimulated much speculation and many experiments. For our purposes here, this problem may be approached from the standpoint that the causal agent

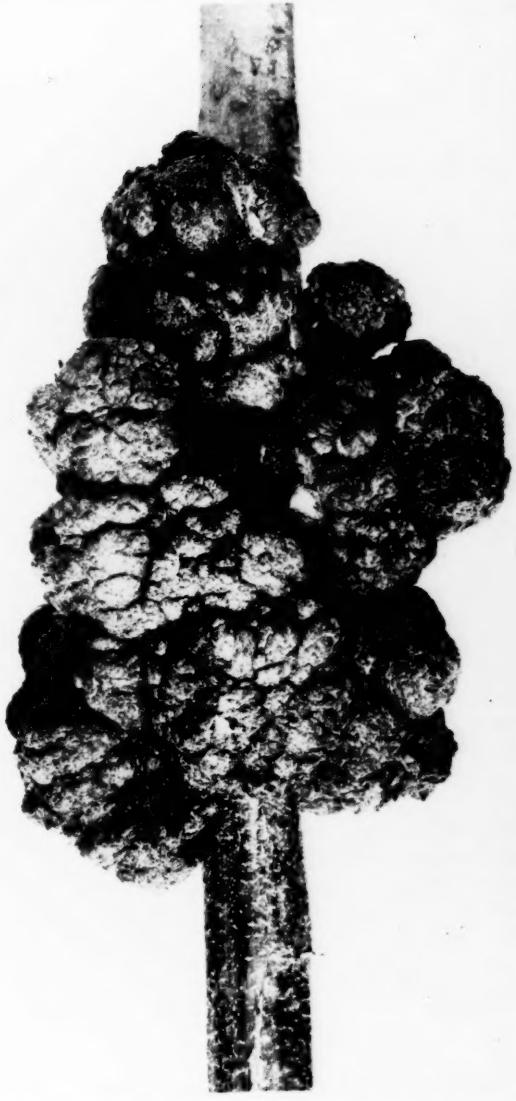

FIG. 1.—Crown gall on field grown Giant Russian Sunflower about three months after puncture inoculation.

starts off the diseased development. This initial agent may or may not be necessary to keep the diseased growth going. One might consider that the causal agent operates as a "trigger" mechanism. However, a trigger alone is not enough. The gun must be loaded. The amount of the load, the character of the load, the amount of dampening the load carries, and so on are also important. Detailed data on the metabolism of the plant, of the causal agent, and of both together are necessary.

Among the different kinds of diseased growth in plants, much of the critical research has been done with crown gall and its causal agent, *Agrobacterium tumefaciens* (Smith & Town.) Conn. For this reason, the rest of this chapter deals chiefly with some important aspects of this complex crown gall problem. A large crown gall that developed on sunflower in the field following artificial inoculation is shown in Figure 1. The literature is so extensive that we can mention only a part. Much of the early work already has been covered by Riker & Berge (72), Levine (47), Grieve (29), Riker, Spoerl & Gutsche (80), and others.

Factors Affecting Crown Gall Development

The host range of the crown gall bacterium is unusually wide (57). It includes most of the dicotyledonous plants with cell sap that is not too acid. The symptoms are much more severe in some species than in others. Particularly susceptible in nature are such plants as apple, peach, plum, raspberry, blackberry, rose, Paris-daisy, and geranium. Common plants used experimentally include mallow, tomato, sunflower, periwinkle, marigold, and carrot. The extent of the host range suggests that the pathogenicity of this bacterium involves rather common and fundamental metabolic processes.

While crown gall is a disease having considerable economic importance (78), particularly on rosaceous hosts, we are concerned here more with the basic interaction between the bacteria and the host cells. This problem perhaps can be visualized best by reference to Figure 2. Here the bacteria were introduced into an intercellular space between three cells which ordinarily would never have divided again. However, under the influence of the bacteria, all three surrounding cells have divided. Several questions appear. What starts off these diseased cell divisions? What keeps them going? In the literature one finds many ideas [reviewed by Riker & Berge (72)]. At least once a year a colleague appears with a new issue of a learned journal and says, "Well, I see the crown gall problem has been solved!" We reply, "What, again?" In general, the ideas fall into two main categories; viz., (*a*) a stimulating factor has been added, and (*b*) an inhibiting factor has been removed.

This review considers the problem from the standpoints of (*a*) the metabolism of the bacteria in culture, (*b*) the host responses, and (*c*) the crown gall and related tissues as they grow in tissue culture.

Bacterial Metabolism

As the crown gall bacterium develops in suitable culture media, a number of changes both physical and chemical occur there. Knowing what happens in such media may help to clarify the action of the bacteria as they work within host tissue.

Physico-chemical factors.—Among the physico-chemical changes, the modification of the hydrogen-ion concentration in one direction or the other is sometimes rather striking, depending upon the composition of the

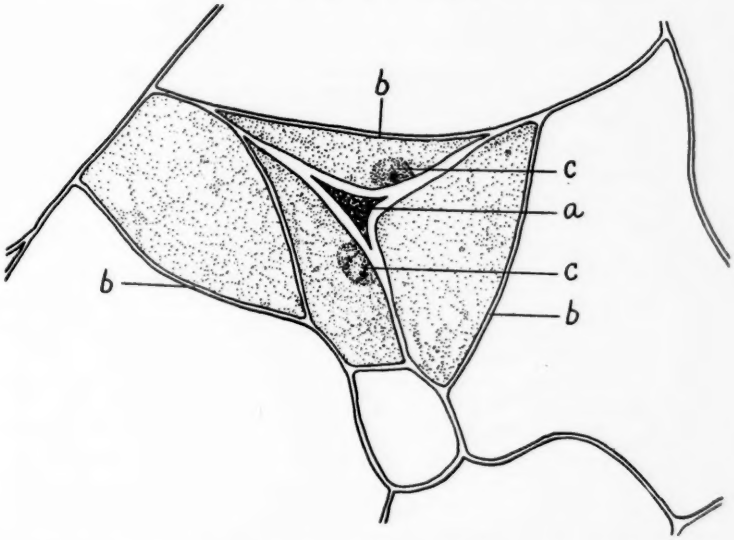

Fig. 2.—The formation of the first crown gall cells. a. The bacteria in an intercellular space. b. New walls laid down to form the new gall cells, which are stippled. c. Nuclei in a position near the bacteria.—After Riker (70).

medium (30, 81). If, for example, ammonium sulphate is employed as a source of nitrogen, as the ammonium is used the pH moves in an acid direction. If sodium nitrate is added, as the nitrate is used the pH moves in an alkaline direction. In the presence of plant tissue extracts, it moves in an alkaline direction (67), presumably because of the utilization of some protein or protein derivatives, with the production of ammonia.

The oxidation-reduction potentials in aerobic cultures drops within a day or two from perhaps +500 mv. to +100 or even to −100 mv., depending upon the medium and circumstances (30, 64, 81).

The osmotic pressure of the medium decreases gradually (79). This presumably is caused primarily by the utilization of sugar which is converted

either into bacterial gum with a large molecule or into carbon dioxide with little, if any, significant accumulation of intermediate products (14, 43, 54).

The surface tension of the culture decreases (1) with the accumulation of bacterial gum which gives the entire culture a conspicuously viscous character. The character of this gum will be considered later.

These and other related changes also accompany the items listed under chemical influences.

Chemical factors.—A considerable variety of chemicals have been introduced into the culture media. For the most part the crown gall bacterium is able to use an unusually large number of different sources of carbon and nitrogen. The details are given by Sagen, Riker & Baldwin (81) and Pinckard (64). Favorable conditions for sugar fermentation were described by McIntire, Peterson, & Riker (54). Likewise this bacterium tolerates many kinds of inhibiting substances (30). Perhaps its ability to grow on so many materials and its resistance to unfavorable substances help to explain part of the reason for the wide host range.

The metabolic products known to be formed by crown gall bacteria have thus far been surprisingly simple (13, 14). In the medium itself the most conspicuous product is carbon dioxide. No volatile organic material has been detected. The change in pH in the acid direction with well-balanced media is usually so small that it can all be accounted for by the presence of carbon dioxide. By far the most common residual metabolite is a bacterial gum (42). In culture its weight is considerably greater than that of the bacterial cells. This gum contains approximately 24 glucose molecules. Braun's results (6) have indicated that the "tumor-inducing principle," or something associated with it, has a large complex structure, approximating that of a protein. The gum is viscous, hygroscopic, and chemically inert. Apparently neither the bacteria nor the host plant has an enzyme system capable of attacking it. When tomato cuttings were placed in water containing gum, it accumulated in plant tissues in sufficient quantity to cause injury and death (43). The margins of the leaves were injured first. The action seemed to be physical rather than chemical. Some polymerized materials having similar molecular weights have proven toxic in direct relation to their molecular weight (40). Since lowered viscosity and swelling have been associated with diseased tissue, this material in sublethal quantities deserves more study in relation to gall formation. Furthermore, the effects in the transpiration stream and between the cells might be quite different. In the intercellular spaces the bacteria and gum fill certain spaces (44, 66). No doubt they interfere with gaseous exchange.

The metabolites resulting from nitrogen in the medium have received much less attention. Ammonia was one of the first products reported (83) and has come the nearest to being a common factor of any found among the different cell-stimulating bacteria (80). When protein derivatives have been used as carbon sources, the pH has commonly shifted in an alkaline direction. However, when sufficient sugar was present it had a sparing action upon the source of nitrogen (14).

The crown gall bacteria have been found to produce biotin, riboflavin, pantothenic acid, and thiamine (55). Since they grow in synthetic media they produce any other such material that is necessary for their metabolism. Locke, Riker & Duggar (52) reported the production of growth substance from peptone broth that contained tryptophan. This was probably a precursor for 3-indoleacetic acid.

An analysis of large quantities of the crown gall bacteria by Geiger & Anderson (26), by Velick (89, 90), and by Velick & Anderson (91) has revealed the presence of various lipids. These are more or less toxic when strong preparations are placed upon host plants.

An attenuated sister culture which has appeared spontaneously among the progeny of the original bacterial culture (30) has provided an interesting tool for determining whether or not specific differences might be correlated with pathogenicity. Comparisons have been made between the virulent and attenuated cultures. In ordinary bacteriological characteristics they have appeared identical. However, there are certain differences. The virulent culture seems more viscous than the attenuated culture on both solid and liquid media (1). Both cultures grew well on L-tryptophan, but the virulent culture is inhibited more by D-tryptophan than the attenuated culture (41). Both produce comparable amounts of auxin in the presence of tryptophan (53).

The attenuation of the virulent culture by means of certain amino acids and related compounds has an interesting bearing upon this problem. The cultures were grown in media with a relatively alkaline reaction and in the presence of enough glycine (about .02 *M*) to reduce but not to stop growth. After a series of 15 or more successive transfers made at several-day intervals, the cultures gradually lost their capacity to induce gall formation (88). Since the original and attenuated cultures were started from mechanically picked single-cell isolations, the possibility was avoided that this process had merely separated the components of an original mixture. In some cases, if the attentuation was not carried through too many transfers, virulence was restored by a series of transfers on ordinary media. In other cases where the cultivation on glycine was carried several transfers beyond the point of attenuation, such restoration did not occur in four years.

A restoration of virulence has been accomplished also by irradiating partly attenuated cultures with ultraviolet light so as to kill all but one in a thousand. The survivors commonly showed a conspicuous increase in virulence (20).

Plant Responses

The morphological responses of the plant tissue to crown gall isolations as they appeared at two-day intervals were described by Riker (65, 66). The wound that introduced the bacteria flooded the intercellular spaces and provided a culture medium for the bacteria. The cells around the bacteria enlarged within two days and the adjacent cell walls turned somewhat brown and took ordinary stains more intensely than normal walls. Within

four days new crown gall cells had formed. In the early stages of development the new cell walls were laid down in somewhat the same manner as those from a wound (68). However, these histological studies were made over a sufficient length of time so that there was no question about whether the cells were wound cells or gall cells. The actual wound through which the bacteria were introduced, except for watersoaking the intercellular spaces, did not influence the cells several millimeters away which helped to form the galls. The morphology of crown gall is described elsewhere (80). This includes evidence on the position of the bacteria between the cells and on the formation of the secondary tumors either by expansion of an inoculated, condensed bud or by movement of the bacteria through channels of liquid. Other reviews also are cited, so that evidence along this line is omitted here.

This four-day interval was determined independently by Braun (3) who inoculated periwinkle plants and subsequently reported that he had killed the bacteria by exposing the plants to high temperature. Plants heated after three days developed only small galls but those heated after four days went on to develop galls which were free from the crown gall bacteria.

The heat treatment of periwinkle provided such a valuable tool that it was further examined by Theis, Riker & Allen (86). They showed that the bacteria would live as long as the plant through the treatment indicated if the relative humidity of the atmosphere were 50 per cent or lower. The bacteria could be present and missed if the cultures of the tissue were made from the surface (the usual technique) rather than from deep in the interior. When the humidity was high enough to inhibit the development of the crown gall bacteria, *Penicillium* sp. grew over the points of inoculation. This fungus produced in culture an antibiotic that destroyed crown gall bacteria. Brown & Boyle (11) reported curing crown gall with *Penicillium* products. The question arose whether this fungus could have led to a confusion of antibiotic killing with heat killing—especially since Braun used Patel's selective medium which inhibits contaminants like *Penicillium*. However, when Theis, Riker & Allen (86) ran the necessary critical experiments that took into account the proper temperature, the high relative humidity, cultivation of tissue from the proper location, and exclusion of contaminating *Penicillium* sp. by aseptic technique, they found, as Braun claimed, that the crown gall bacteria could be destroyed by heat without killing the tissue.

The old ideas about autonomous growth, once disease in the tissue was started, thus have received experimental evidence with *Vinca rosea* L. This brings closer still the comparison between crown gall and certain disease growths in animals. The relatively complete enzyme system demonstrated by this periwinkle, as discussed later, makes this plant a particularly favorable one for study. How frequently autonomous growth also occurs in other plants remains uncertain. The failures of many "secondary" crown galls on sunflower, Paris-daisy, and marigold to continue development after the bacteria have died out have suggested caution about the broad inferences sometimes made from this evidence.

A favorable temperature for the development of crown gall was studied earlier on tomato, apple, and various other plants (67, 75, 79). For example, crown gall developed well on tomato plants that were growing vigorously until the temperature rose to 28°C. Although at 30°C. bacteria grew well in culture and tomato plants grew well in soil, galls did not develop. This sharp temperature differential, reported by Riker in 1926, and repeatedly confirmed, has provided an excellent tool for further studies on pathogenicity.

The critical temperatures for gall formation have been examined recently to determine the incubation period required. A temperature of 32°C. during the four days immediately following inoculation prevented gall formation (4) on *Vinca rosea* but galls developed on plants held for four days or less at 32°C. Virulent bacteria were present in the first case because rewounding the old inoculations and placing the plants at 20°C. was followed by typical gall formation. Braun (5) also reported that plants inoculated and incubated for five days at 32°C. and then heat treated (47°C. for five days) to kill the bacteria failed to develop galls. Similarly treated plants held at 26°C. for five days and then exposed to 47°C. for five days all developed galls. Later experiments of this type indicated that the first evidence of gall formation was found after exposure of the plant tissue to the bacteria for about 30 hr. Furthermore, the changes in the periwinkle cells occurred at temperatures below 32°C. during the four-day period immediately following wounding. As with periwinkle, Braun & Mandle (8) found that the bacteria require an incubation period as cool as approximately 25°C. to produce galls on *Kalanchoe diagremontiana*. Furthermore, the size of the galls was proportional to the total duration of exposure to 32°C. when this temperature was alternated with 25°C. for an accumulated period of 30 hours at 25°C. This suggested that the activity of the transforming principle was destroyed at 32°C. at roughly the same rate as it was produced at 25°C.

The thermodynamics of this situation were studied by Braun (6) with the *Kalanchoe* plant by applying the Arrhenius formula to the progressive inhibition of crown gall development between 27.5 and 28.8°C. Activation energy of more than 80,000 calories per mole was calculated. Such reactions were considered characteristic of protein denaturation. They suggested either that the tumor-inducing principle itself or something intimately associated with its inactivation is a factor of complex structure.

The differences in chemical composition between the galls and correspondingly healthy tissue have been examined by several different workers. For example, proximate analysis indicated that the gall tissue resembles that of young plants, being high in nitrogen and low in fibrous material (59). Considerable variations were observed depending on the time of collection and on the species of plant. The actively growing tissue contained more ascorbic acid. An increase of thiamine appeared in the gall tissue within a week (31).

The enzyme content of the galls was different from that of the healthy tissue. The galls contained 86, 73, and 57 per cent more than the stems

of catalase and peroxidase (59) on a total nitrogen basis. The galls had active tyrosinase, while the stems had little, if any. Furthermore, the galls contained relatively much more glutathione than the stems.

Reduced respiration levels by the gall tissue deserve attention. With the considerable excess of such oxidizing enzymes, the suggestion has appeared that the basic metabolic activity of the galls is relatively anaerobic in comparison with that of the neighboring tissue. This condition deserves study as a causal factor for cell stimulation. It may be correlated perhaps with the earlier observations that the tissues involved had flooded intercellular spaces. The diffusion of oxygen through normal tissue is greater than that in tissue with water in the intercellular spaces (28). Goddard (28a) estimates that it is 2,000 to 3,000 times as fast with intercellular spaces of 3 per cent by volume. As the amount of hyperplastic tissue increases in size, more difficulty with gas exchange doubtless also develops. The crown gall bacteria lower the oxidation-reduction potentials of the material in which they are growing. The formation of ammonia and the consequent change of the pH in an alkaline direction also lowers the oxidation-reduction potential. The bacteria and gum block certain intercellular spaces. The presence of gummy materials, which are hygroscopic, might cause the cells to swell and to metabolize more slowly. The reduced oxygen uptake (56) in the presence of 3-indoleacetic acid at a gall-inducing concentration has added interest, as discussed later.

Among the more important of the growth substances is the plant hormone 3-indoleacetic acid. Alpha-naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid are still more active. Among the well-known growth substance effects are increased epinasty, adventitious roots, cambial activity, bud inhibition, and delayed abscission. All were found (51) associated with crown gall on tomato. The hormone in tomato crown gall was either 3-indoleacetic acid (53) or a substance similar in its effect on *Avena* coleoptiles and its sensitivity to acid and alkali. When applied to a cut stem in a slowly available form, as when it was dissolved in lanolin, 1 per cent 3-indoleacetic acid induced chemical galls (12). Their structure resembled that found in the crown gall (46). Link & Eggers (50) reported much more auxin in galls than in stems. From these and other data the concept developed that the chemical cause of crown gall had been demonstrated. Various reasons for caution about such a view were reviewed by Riker, Henry & Duggar (74) and Mitchell, Burris & Riker (56). Particularly, data based on wet or dry weights have failed to consider the inert wood in certain stems. However, the presence in galls of something like 3-indoleacetic acid at approximately 6 to 12 μg. per l., an amount comparable to that in some actively growing and normal plant parts, has been amply demonstrated. While these extremely small amounts strikingly affect certain tissues, much stronger and almost lethal concentrations are needed to induce chemical galls.

The stimulation of tissue about inoculations with the attenuated crown gall bacteria has been possible not only with the virulent culture higher on

the stem (51) but also with galls induced by certain chemicals. Braun & Laskaris (7) indicated that the growth substances used served merely to stimulate cells previously altered by the attenuated culture. Riker (71) has suggested two possibilities. The chemical might have done it, but also the callus tissue formed by the chemical might have supplied the missing factor. Thomas & Riker (87) examined a large number of chemicals including the common plant hormones for the stimulation of attenuated bacterial galls. They found no correlation between the formation of these chemical galls and any other growth substance effects.

Various related topics were treated at the Wisconsin symposium on growth substances (82).

Some effect on respiration by large amounts of growth substance seems possible. This is in line with the ideas mentioned earlier that the reduced respiration levels were important factors associated with gall development. At the concentration necessary to induce gall formation, all the growth substances tried reduced respiratory activity (56). 3-Indoleacetic acid at 55 mg. per l. reduced that of tomato stem slices 55 per cent at pH 5.0. At pH 6.0, 400 mg. per l. of this chemical reduced it 50 per cent. Chemicals having an aromatic ring and free carboxyl group were most effective. Some other structurally similar compounds, e.g., benzoic, salicylic, and nicotinic acids, also inhibited respiration. The system inhibited by growth substances was also limited by low oxygen tension. The enzymes oxidizing cytochrome-*c*, ascorbic acid, catechol, and glycolic acid were not affected. Decarboxylation of pyruvate under anaerobic conditions was affected little, if any.

The possibility of studying the physiology of these diseased tissues has been greatly enhanced by the development of the tissue culture techniques. Following basic experiments by various other workers, White (92, 94), Gautheret (23), and Nobécourt (62) successfully established true plant tissue cultures. These consisted of masses of largely undifferentiated callus that grew indefinitely on synthetic media [White (94)]. Gautheret (24) and Morel (58) have reviewed the literature and have described the results with tissue cultures from many species. Thus we have an important tool to determine which substances the tissues can use, which are not available, and which are inhibiting and at what concentrations. Such determinations have been undertaken in an extensive series of studies with tissue cultures.

Results from Tissue Cultures

The diseased tissues in culture have been derived most commonly from crown gall that was free from bacteria, or from growths having a comparable origin. To secure such cultures, a large number of tissue isolations were made from primary and secondary galls. Many of these contained bacteria, but some were free of bacteria as also were certain portions of primary galls. Among the bacteria-free cultures only a few grew satisfactorily, but enough so that the cultures could be increased and carried indefinitely on synthetic

media. Cultures of bacteria-free tissue from secondary crown galls on sunflower were first reported by White & Braun (95). They also showed in these and later reports that the tissue free of the bacteria was capable of unlimited growth *in vitro* on synthetic media. Primary and secondary crown gall tissues from a number of genera free of the inciting bacteria are now growing freely *in vitro*.

The extensive literature on root tip cultures, embryo cultures, and so on is considered elsewhere.

Physical conditions.—The optimum temperature for sunflower tissue of crown gall origin growing *in vitro* is 24° to 28°C. That for tobacco stem tissue from the hybrid *Nicotiana glauca* by *N. langsdorffii* is about 32°C.

The best final acidity for tobacco tissue is pH 5.5 to 5.9 and for sunflower is pH 6.0 to 6.4.

The best sucrose concentration for sunflower and tobacco tissue on White's medium is about 1 per cent (37). The influences of the kind of carbohydrate and of the concentration are considered more fully below.

Plant and bacterial products.—Media fermented by crown gall bacteria, from which the bacteria were removed, inhibited the growth of sunflower tissue, especially at high concentrations. Lyophilized crown gall bacteria at high concentrations also inhibited growth.

Extracts of crown galls on tomato or marigold, and of yeast stimulated growth when dilute, and inhibited growth when concentrated. The effects were more evident on sunflower tissue, which grew more rapidly, than on tobacco. An extract made from crown gall on Paris-daisy stimulated sunflower tissue and inhibited tobacco tissue (39). These and other similar results have indicated the need for a study of basic nutrients for an understanding of growth with complex materials.

Minerals.—With White's medium (93) as a basis, the best concentrations of mineral salts and of certain vitamins were studied with 108 different media. A modified triangular method was employed with geometric progressions. An estimate was given of the effects on growth from increasing or decreasing not only all the nutrients in the basal medium but also ferric tartrate, nicotinic acid, and pyridoxine. Basic media were developed (38) that considerably increased the growth secured.

Growth substances.—Materials such as indole, naphthalene, naphthoxy, and phenoxy compounds were used with sunflower and tobacco tissues. Commonly the wet weight of sunflower tissue was increased at low concentrations (10^{-7} or 10^{-11} gm. per l) and decreased at higher concentrations (10^{-3} gm. per l). Dry weight followed wet weight closely (32). Histologically the sunflower tissue had larger cells with high concentrations of the growth substances. More scalariform tracheal elements appeared in tissues grown in low concentrations (84).

The role of a growth-regulating substance in normal tissue growing *in vitro* has received extensive treatment from Gautheret (25) and Nobécourt (63) and their associates and need not be reviewed here.

The role of growth-regulating substance in tissues both of normal and of crown gall origin has been examined. Crown gall tissue from sunflower and *Vinca* either was little affected or was inhibited by low or high concentrations respectively of growth-regulating substance according to de Ropp (15). However, normal tissue of the two species produced many roots at low concentrations and was little affected or inhibited at higher concentrations. He reported no gross structural changes as a result of auxin action. In later studies, DeRopp (16) presented evidence that crown gall tissue *in vitro* generated a substance capable of stimulating proliferation of normal callus tissue. By means of *in vitro* grafts he found that tumor tissue brought about proliferation of normal tissue to which it was grafted. However it seemed difficult, if not impossible, at this stage to determine exactly which was the normal and which was the tumor tissue. Most recently, DeRopp (17) has examined the response of carrot and artichoke tissue to the crown gall bacteria, to 3-indoleacetic acid, and to both. Bacteria applied to the surface of the tissue slices induced galls in each case, but the application of 3-indoleacetic acid produced only small or no overgrowths. Levine (49) examined the effects on growth of tissue by 3-indoleacetic acid and of some common chemical carcinogens. No toxic effects were observed with the carcinogens at concentrations from 1 to 190 mg. per l. Sometimes stimulation of callus and root formation appeared. Braun & Morel (9) made comparisons between bacteria-free tissue cultures from grape of crown gall, habituated callus, and callus. Each type was grafted back into grape. Respectively, they grew well, moderately, and poorly. Although the habituated tissue, like crown gall, synthesized 3-indoleacetic acid, the two tissues were still quite different.

Nitrogen sources.—The kind and amount of nitrogen were often critical. Sunflower tissues grew excellently with nitrate and urea as sole sources of nitrogen on basic media with sucrose and suitable mineral salts. Good to poor growth developed with ammonia, alanine, glycine, arginine, asparagine, and aspartic acid, as well as with casein hydrolysate, peptone, and yeast extract. Eleven amino acids permitted no growth. When nitrate was present to allow excellent growth, such growth was gradually inhibited by increasing amounts of ammonium salts and arginine. Growth was sharply inhibited by nitrite, asparagine, and all the amino acids tried—in most cases near the critical 0.001 *M* and stronger concentrations. However, growth developed with stronger (0.064 *M*) concentrations of alanine, aspartic acid, and glutamic acid, perhaps because of transamination.

Various nucleic acids and related compounds were utilized by the virus tumors of sorrel in culture (61). Growth was enhanced by the addition of alkaline-hydrolyzed ribonucleic acid in the range of 0.2 to 0.8 mg. per ml. and decreased by comparable levels of alkaline-hydrolyzed desoxyribosenucleic acid. Marked inhibition was observed in media containing adenine, adenosine, or adenylic acid. Some evidence of stimulation was observed in media containing guanine, uracil, xanthine, or hypoxanthine. At concentrations of 0.3 or 0.4 mg. per ml. of medium, 6-ketopurine (hypoxanthine) was

stimulatory; 2,6-diketopurine (xanthine) less beneficial; and 2,6,8-triketopurine (uric acid) definitely toxic. The triazolo analogue of guanine was inhibitory at all levels tested down to 0.001 mg. per ml. This inhibition was progressively reversed by guanine added over a range from 0.01 to 0.30 mg. per ml.

Various sources of nitrogen deserve more study, particularly as growth-stimulating, inhibiting, or regulating substances (73).

Carbon sources: sugars and polysaccharides.—The source of carbon was studied with tissues from marigold, Paris-daisy, periwinkle, sunflower, and tobacco. The tissues generally were able to grow on a wide variety of common sugars and polysaccharides as a carbon source at 1 per cent concentration. All grew more or less on dextrose, levulose, sucrose, maltose, cellobiose, dextrin, or pectin. Some species grew well and others poorly or not at all on mannose, galactose, lactose, raffinose, or starch. All species grew poorly or not at all on arabinose, xylose, rhamnose, or inulin. None of any species grew either in the light or in darkness in the absence of carbon (33). The tissues developed no chlorophyll.

The concentration of the carbohydrates was often a critical item for growth (34). The five species were tested with different concentrations (8, 4, 2, 1, .5, .25, .125, .06, .03, and .015 per cent) of each of the following: dextrose, levulose, mannose, galactose, sucrose, maltose, lactose, cellobiose, raffinose, starch, dextrin, and pectin.

Marigold tissue had the greatest wet weight on media containing 2 per cent dextrose and progressively less with 2 per cent cellobiose, 4 per cent levulose, 2 per cent sucrose, 4 per cent maltose, or 4 per cent raffinose. Paris-daisy tissue had the greatest wet weight on media containing 4 per cent levulose and progressively less with 4 per cent maltose, 4 per cent sucrose, or 1 per cent dextrose. Periwinkle tissue had the greatest wet weight on media containing 2 per cent raffinose and progressively less with 1 per cent levulose, 1 per cent galactose, 2 per cent dextrose, 1 per cent sucrose, 2 per cent lactose, 1 per cent maltose, 2 per cent cellobiose, or 1 per cent dextrin. Sunflower tissue had the greatest wet weight on media containing 2 per cent dextrose and progressively less with 2 per cent sucrose, 4 per cent cellobiose, 0.25 per cent levulose, or 2 per cent maltose. Tobacco tissue had the greatest wet weight on media containing 1 per cent levulose and progressively less with 2 per cent dextrose, 2 per cent maltose, 2 per cent cellobiose, 2 per cent sucrose, 4 per cent raffinose, or 4 per cent dextrin. Although all species grew well within a certain range of concentration with a few compounds, certain species grew well over a wide range with many of the compounds, and some species grew poorly if at all at any concentration.

Alcohols.—The alcohols alone, generally at 0.5 per cent, were not good sources of carbon. However, in the presence of sucrose all species grew more or less on media containing methanol, ethanol, glycerol, erythritol, mannitol, or dulcitol. Butanol was tolerated at this level only by periwinkle, and phloridzin only by marigold and sunflower. None of the species tolerated

propanol (33). The concentration of alcohols with and without sucrose was important in the amount of growth of the tissues (35).

Organic acids.—The organic acids (adjusted to pH 6.0 with sodium hydroxide) at 0.5 per cent were generally poor sources of carbon alone. However, when tested in addition to the sucrose, many were tolerated. All species grew more or less in the presence of stearic, pyruvic, malonic, succinic, fumaric, malic, or tartaric acids, or calcium gluconate. All grew poorly or not at all on glycolic, lactic, oxalic, glutaric, or citric acids. None grew in the presence of formic, acetic, or propionic acids (33). The organic acids at concentrations from .015, .03, .06, .125, .25, .5, 1, 2, and 4 per cent failed to support growth alone. However, in the presence of sucrose, certain compounds stimulated or inhibited growth, depending upon the concentration employed in the nutrient (36).

With tissue from virus tumors of *Rumex*, Nickell & Burkholder (60) have reported more or less parallel studies. Various interesting parallels and differences appear.

Other items.—These tissue culture studies have shown some striking differences between the tissues from different species. Are we opening further the door for physiological as well as morphological understanding of tissues? In any case, many interesting possibilities appear for studying various aspects of plant diseases.

The possibilities of comparing diseased with healthy tissues in cultures present some interesting opportunities and difficulties. The callus tissues, whether from an injury or a bacteria-free piece of crown gall, are strikingly similar in many ways. One wonders whether he is justified in calling the callus from an injury a normal tissue. In any case, perhaps some of the basic metabolic activities can be worked out most simply with the gall material that grows so well. Comparisons can then be made in relation to the items that have appeared most important.

Growth inhibition from certain amino acids and organic acids has led to the hope that still other and more powerful agents would soon be found. DeRopp (18) tested 57 different substances. The most active inhibition came with analogues of pteroylglutamic acid. These inhibited callus growth at 10 to 100 μg. per l. The 4-amino-N^{10}-methylpteroylglutamic acid applied locally to young crown galls on sunflower at 0.1 mg. per ml. completely inhibited gall development. Certain nitrogen mustards, 8-azaguanine, and cortisone also inhibited crown gall. Dimond (19) has inhibited crown galls by means of continuous gamma irradiation.

In conclusion, many trigger agents have been associated with diseased plant growths. Whether they operate by providing stimulation or by removing inhibitors remains to be determined. However, as mentioned earlier, the trigger is not enough. What happens depends on the kind of load, the amount, and whether it is dampened by inhibitors. The use of tissue cultures has opened the way for determining much about the load, about dampening materials, and about the amounts of both necessary for effectiveness. Basic

information is being developed not only for the influence of common mineral salts, sources of carbon, sources of nitrogen, and various metabolites, but also for concentrations, respectively, that encourage or inhibit growth. Such data seem fundamental for estimating the interplay of factors that influence the manner and kind of growth.

The idea that diseased growth develops from an imbalance of critical factors (71) fits well into this concept. While we shall continue to analyze individual factors that by their presence or absence may change normal into pathological growth or keep it going, no one thing may be responsible. For normal growth, a number of factors operate in a suitable balance. However, in pathological growth of one kind a group of these factors may be out of balance. Likewise, in pathological growth of another kind, the balance may be disturbed in some other way.

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VIRUS-INDUCED TUMORS OF ANIMALS[1]

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Introduction

While the concept is not new, appreciation of the fact that viruses or virus-like agents may possess the ability to elicit growths in animal tissues is entering the thinking of an ever-widening circle of modern oncologists. The importance of these observations and the diversity of literature on the subject warrant at this time a review of the studies which have contributed to the development of this field. The present article does not attempt to be complete in its coverage; on the other hand, effort has been made to evaluate certain significant findings, and to bring together in one discussion observations which at first might seem irrelevant but actually may be closely related. Limited space has necessitated the selection of particular growths for detailed discussion and the casual consideration or even neglect of others. This is no reflection upon the importance of the latter. The references chosen obviously cannot be all-inclusive, but rather are designed to give the reader an opportunity to approach the literature with the assurance that he will find further references in the articles listed.

Virus-Induced Tumors of Invertebrates

It is questionable if the terms applicable to growth phenomena at the vertebrate level can be transferred with impunity to the invertebrates. Cancer and malignancy connote vertebrate concepts, but tumor is a general enough term to have meaning when considering the latter group. However, in the *Insecta* it is sometimes difficult to decide whether a tumor is truly pathological since certain organs, notably the fat-body, normally increase in size when infected by microorganisms.

Steinhaus (127) has classified the viruses, which are known to infect insects, on the basis of their ability to produce polyhedral inclusions in the infected cells. Bird (11) has described a polyhedral disease in the European spruce sawfly, *Gilpinia hercyniae*, associated with an abnormal proliferation of the mid-gut epithelium. The regenerative cells located just beneath the basement membrane become infected with the virus. As a result, the natural process of epithelial replacement during moulting is accentuated, and tumors are formed which may project into the lumen of the gut or into the body cavity. Large growths are found only when larvae become infected just before the last moult. If the larvae are infected earlier, they die before the tumors become too evident. On the other hand, if general infection takes place after the adult gut is formed, the effect of the virus is inhibited until after pupation. Histologically, the mature growths contain a center consist-

[1] The survey of literature pertaining to this review was concluded in March, 1951.

[2] It is a pleasure to acknowledge the assistance of Mrs. J. C. Cunningham in checking the bibliography.

ing of a necrotic, pigmented mass of epithelial elements surrounded by a thin layer of proliferating cells. Bird feels that these growths are not malignant.

Paillot (97) has described two diseases in the cutworm, *Euxoa segetum*, which are associated with changes in the adipose tissue of the larvae. He refers to them as "pseudo-grosserie 1" and "pseudo-grosserie 2." These are not polyhedral diseases, but infected cells possess characteristic small coccoid granules which Paillot feels represent virus bodies. In disease 1, the infected fatty tissue proliferates more than normally and may contain follicular nodules. In disease 2, cells of the fat-body, trachea, and hypodermal tissues are involved and contain these granules also. The infected cells undergo considerable proliferation, and Paillot feels that particularly disease 2 may resemble neoplasia as seen in higher forms.

While many other growths have been described in the invertebrates, their nature and the possibility of their virus etiology must be most carefully elucidated. There are many opportunities for further studies and important contributions using invertebrate material. Recently, Scharrer & Lochhead (113) have reviewed the literature concerning tumors in the invertebrates.

Virus-Induced Tumors in the Lower Vertebrates

What constitutes proof that a virus is present or that it induces a tumor in these forms is difficult to say. Most of the evidence for the existence of an agent is based upon two lines of approach: (*a*) the presence of cell inclusions, and (*b*) the ability to infect normal animals by placing them in water in which individuals with tumors are, or have been swimming. Possibly a third criterion might be the failure to isolate specific bacteria from the growth, a procedure that has not frequently been attempted. The weaknesses of these criteria are obvious. In spite of these difficulties, the concept of virus-induced tumors in fish, for example, is not new. The virus etiology of papillomas in fish was suggested in 1908 (82).

Weissenberg (140) has recently reported further studies of lymphocystis disease in fish. There is a question if the typical lesion of this disease should be considered a tumor in the sense in which we are now using the term, since histologically the main effect is the result of an increase in cell size rather than cell number. At any rate this most interesting disease is associated with cytoplasmic inclusions and further work is in progress to determine whether they take origin from certain basophilic granules in the host cells.

A spontaneous carcinoma of the skin in *Triton alpestris* described by Champy & Champy (30) has been considered by some to be an example of a virus-induced tumor in newts.

Renal Carcinoma of Frogs

In 1934 Lucké, described growths in the kidneys of the frog, *Rana pipiens*, which showed a variety of histological types ranging from benign adenomas to frankly malignant adenocarcinomas with metastases. The presence within the cells of these tumors of intranuclear, acidophilic, herpetiform inclusions

suggested the possibility of viral etiology. Subsequent work confirmed this suspicion, since Lucké found that cell suspensions of the tumors when inoculated by way of a variety of routes into normal frogs resulted in renal growths with no reaction at the injection site. Histologically, the new tumors were similar to the parent growths and possessed intranuclear inclusions in their cells. Lyophilized as well as glycerinated tumor material gave similar results.

Heterologous transplantation of these growths into the anterior chambers of the eyes of other cold-blooded vertebrates has been possible and as in the homologous transfers there is a definite lag period of 5 to 10 days before growth takes place. This is unlike our experience with the Rous sarcoma, since there no measurable lag period is evident (121). The renal carcinoma will grow in the eye of toads (*Bufo Americanus*), but shows no increase in size in fish or reptiles and rapidly deteriorates in the eye of the alligator. It has been possible to maintain the growth by serial passage for over two years in the eyes of *Rana pipiens*, and in recent transfers there has been a definite increase in the tendency to invade the surrounding tissue of the eye (115).

Briggs & Grant (17) transferred the renal tumor from the frog to the tadpole and observed that the new environment did not alter the growth nor the histology of the neoplasm. If the tumor was imbedded in the tail fin the transplants increased in size until the approach of metamorphosis, at which time they regressed. It is interesting that similar results were obtained if nonmetamorphosing tadpoles, made so by thyroidectomy and hypophysectomy were used. On the other hand, if tumors were placed at other sites, they withstood the effect of metamorphosis and persisted in the adult frog.

In tissue culture, using chicken-frog plasma media, the carcinoma cells extending out from the explant do not contain inclusions even though the original tissue possessed them (83). Whether the absence of the inclusions is an indication of an insufficient time between transfers for their development, or whether the environmental conditions are not right for their expression is not known. One wonders if these cells would produce tumors and if so, does this suggest that the virus may not be necessary for the maintenance of the neoplasm? These marginal cells of the explant often become detached and show amoeboid movement. This tends to increase with rise in temperature.

Temperature likewise influences the growth rate and histology of the frog carcinoma grown in the anterior chamber of the frog's eye. At 7°C. growth is slow and characterized by short stubby tubular outgrowths with few cysts, while at 22°C. growth is more vigorous with extensive vascularization, long branching tubules, and numerous cysts. In frogs possessing kidney neoplasms, temperatures of 28°C. for 50 days resulted in metastases in 54 per cent of them, in contrast to only 6 per cent in those held at 7° or 18°C. (84). At the higher temperature, the metastatic lesions were more widespread as well as more numerous, and since they were nearly the same size,

Lucké & Schlumberger suggest that they probably were about the same age. It was further noted in this study that the nutritional state of the frog was important in the instance of metastasis, the better nourished animal bearing the wider dissemination of growths. Under these experimental conditions, the frequency of metastatic spread was not correlated with the size of the primary tumor, nor did increased temperature seem to influence the size of this primary lesion (82).

By treating anterior chamber transplants of the renal carcinoma with x-ray (85), it has been found that this growth responds in a manner similar to that of carcinoma in warm-blooded animals. With small divided doses, a larger amount of radiation is necessary to produce the same effect that a single large dose might produce.

Biochemical studies (82) have been undertaken on this frog tumor and it has been found that as the neoplasm grows and becomes more invasive, the alkaline phosphatase decreases and as time goes on may finally disappear. On the other hand, the acid phosphatase increases with tumor growth, and metastases from older carcinomas are also high in this enzyme. The significance of these findings is not clear at this time.

Schlumberger (116) has studied the effect of methylcholanthrene on the incidence of renal carcinoma in the frog. The results indicate that the carcinogen was not the initiating factor in the neoplasms observed since they were considered to be spontaneous, and that the chemical did not activate the virus known to be latent in the tissue. This latter point is of interest in the light of the findings of other investigators working with rabbits (108).

Chicken Tumor Number 1 of Rous

In 1911, Rous described a sarcoma of chickens which was transmissible by cell-free filtrates. Since this time a voluminous literature has developed covering almost all phases of the tumor and its etiological agent.

There are many points regarding the epidemiology of this disease which are not known. The sources and modes of infection are still to be elucidated. It has been the experience of most investigators of the Rous tumor that normal birds mingling with infected ones do not develop growths as a result of this contact. In fact, pieces of tumors may be eaten by normal birds without deleterious effect. Carr (24) has found that egg transmission of the agent, if it occurs, is probably not an important factor even though the yolk may possess a considerable amount of antibody. Using six-week old chicks, Carr (25) did not observe any seasonal variation in the ability to pass the tumor experimentally. This has not been the experience of all workers, however [see (25)], since other investigators have observed seasonal variation in filtrability of the growths as well as variation in response to chemical carcinogens. From these studies there is no question but that such factors as moulting, laying, nutrition, and age of host all affect the expression of the Rous agent. In some cases, seasonal cycles may coincide with cycles of ectoparasitic infestation, a lead which might be interesting to follow. The genetic composition of the bird likewise has been shown to be important by Carr, who

has selected individuals for resistance to the Rous agent. He also considers that this resistance is not due to the presence of antibodies. Greenwood *et al.* (65) have concluded that resistance is associated with complex genetic relationships. Their tumor resistant strain was also relatively resistant to the action of carcinogens. Further, it is known that the Rous agent may remain latent in birds for long periods of time before it elicits tumors (26), a property which may be important in the epidemiology of infection.

Claude (31), extending the work of Ledingham & Gye, and also McIntosh [see (31) for references], purified the Rous agent by differential centrifugation and found activity associated with particles 70 mμ in diameter with a density of 1.3. Shemin & Sproul (120) used the differential centrifugation technique on tumor extracts which had been previously treated with papain. Their papain-free, purified fractions were homogeneous by electrophoresis at pH 7.4. Dmochowski obtained similar results with the Rous and Fujinami tumors. He followed the virus activity of his fractions by tumor production as well as their ability to fix complement with anti-Rous rabbit serum. Using Rous, Fujinami, and Mill Hill #1, he also tried adsorption and elution of these tumor extracts with aluminum hydroxide, and obtained dialysates with greater activity than the original tumor extracts (38). Riley (105) has used celite for purification of the agent and has shown that while the virus is adsorbed on the diatomaceous earth, it is readily eluted with distilled water or dilute sodium chloride solution. These are only some of the methods that have been developed to obtain relatively pure Rous virus.

The significant and intriguing point found by Claude (32) in his chemical analysis of the Rous tumor tissue and tissue of normal chick embryo is that actually the two differ but little, yet biologically, of course, their activity is most diverse. The two main groups of substances recovered in the purified material regardless of source were ribose nucleoprotein and phospholipids. Properties of the relatively purified agent show an isoelectric point of 3.5 and a pH range giving activity from 3.8 to 11.4. The spectrophotometric absorption in the ultraviolet is at 2575 Å. Recently, Bryan *et al.* (18) have studied the sensitivity of the Rous agent to x-radiation. Using the method of Lea, they have determined that the size of the radiosensitive area is 46 to 49 mμ in diameter. Since the total size is considered to be in the region of 70 mμ, it seems that not all of the particle is sensitive to x-ray. The above authors have pointed out that in the electron micrographs of Claude *et al.* (33) cells of the Rous tumor contain particles ranging from 67 to 85 mμ which are considered to be virus. These particles are composed of a dark central area, which, according to Bryan *et al.*, are 40 to 50 mμ in diameter, surrounded by a paler halo. The relationship between these two observations awaits clarification. A more detailed summary of the chemical properties of the Rous virus may be found in Greenstein's book (64).

The filtrable properties of the Rous sarcoma have been shown to be quite variable. In 1927, Baker & McIntosh (5) suggested that the presence of an inhibitor might be responsible for the non-filtrability of the chicken sarcoma. By treating tumor extracts with trypsin in an acid pH they were able to

render their filtrates active. Claude (34) has pointed out that the inhibitor is a protein, but unlike Carr (27) is reluctant to call it antibody because it has some element of nonspecificity. Because of the practical aspect in the laboratory and the more theoretical implications of the masking of viruses in general, we have been interested in this problem, too (122). Five different non-filtrable tumor lines were established from a second passage filtrate-inactive growth. The original bird bearing the tumor was 22 days old and was killed 20 days after injection. In subsequent transfers, inactive filtrates were obtained from sarcomas in 100 per cent of the birds tested. Some of these lines were carried for 10 or 11 transfers before they were either lost, discontinued, or the tumors became filtrable. Some growths became filtrable on the sixth or seventh passage. The return to positive filtrates was as sudden as the loss of this property. Attempts were made to determine the reason for this phenomenon. Inactive filtrates were mixed with those that were active, obtained from Doctor Duran-Reynals' laboratory, and there was no evidence of any neutralizing effect by our material. Treatment with high frequency sound waves of 9,000 c.p.s. for 35 min., with the idea of destroying any combination between virus and inhibitor, was done without success of separation. Trypsin-treated cell emulsions yielded inactive extracts and filtrates. During this period of non-filtrability, tumor lines could be perpetuated only by cell suspensions, tumor extracts being inactive. It is important to note that this phenomenon was seen in young birds the majority of which were 7 to 14 days old.

Miszurski *et al.* (91) have treated 5 non-filtrable growths with 50,000 r of x-ray and made 3 of the 5 filtrable. Materials were tested in 2 to 6 month old Leghorns. Duran-Reynals (41) describes work with Freire in which they found masking of the agent in 62 per cent of chickens 15 months or older bearing tumors, and in only 17 per cent in chicks two weeks of age In addition to the influence of age of host upon the filtrability of the tumor, the age of the tumor (28), and whether the growth had been transferred by cell suspension or filtrate previously, proved to be important factors.

As suggested by Riley (106), some cases of non-filtrability can be attributable to adsorption of the agent on the filter material. Particularly is this true in growths with low virus content. Riley has calculated that it takes from 1 to 100 virus particles to initiate a tumor, and even in an actively growing sarcoma the amount of virus nitrogen is exceedingly small. That an inhibitor is present in some cases is evident from the experience of many that unconcentrated purified extracts may be more active than the original tumor material.

One of the greatest handicaps to the study of the Rous sarcoma and its agent has been the lack of any accurate way to quantitate the virus. If this were available, the problem of masking would be on little firmer ground. Bryan (19) has pointed out that statistically neither the presence nor absence of growths, nor the relative sizes of tumors reflect accurately the amount of virus injected. On the other hand, the reciprocal of the latent period, that is from the time of inoculation to the first appearance of the tumor, does hold

a linear relationship with the logarithm of the dose of the agent. By a system of rotation of inoculation sites into which experimental and control materials are injected, it is possible to set up a Latin square, and thus control genetic constitution, immunological status, sampling of materials, and variation due to injection into different anatomical locations all at one time (20, 21). Chicks 3 to 10 weeks of age are best for this technique.

Many interesting things have been discovered by the transplantation of the Rous tumor to foreign hosts. Duran-Reynals (42) found that it was possible to infect ducks with the Rous tumor virus if one used large doses and injected them during the first 24 hr. after hatching. In the duck, lesions developed during the first 30 days that were not too unlike those found in chicks inoculated in the same manner. However, lesions which developed after this time, 40 days or later, were of a nature not regularly found in chicken infections. Extracts of tumors appearing early in ducks would not cause lesions in ducks but were active for chickens, while the extracts of the late lesions readily infected ducks, showing adaptation to this species. After establishing several duck strains of the Rous agent, Duran-Reynals reversed the process by injecting the duck variant into day old chicks. Here, as in the ducks, early and late lesions appeared. The early lesions were the result of the duck variant infection and the late lesions yielded virus which was typically chicken Rous type. Among the characteristic features of the altered agent in the chick was the presence in increased number of endosteal and periosteal tumors. The alteration of the virus produced by passage in ducks was dependent upon the age of the chicken from which the original virus was taken (41, 54). Duran-Reynals has described three other duck variants (43) which possess characteristics other than the ones mentioned above. One variant produced local tumors in 12 month old pigeons; another had an affinity for the cerebral vessels in young ducks (44). The histopathology of the latter infection suggests that the virus acts directly on the vascular endothelium causing the cells to swell. With the loss of integrity of the vessel wall, weakness results and rupture takes place under the continuous pressure of the blood. Other perivascular tissues are also altered by the agent. These are much the same lesions described by Duran-Reynals (45) in young chicks in which the agent has produced hemorrhagic disease instead of tumor formation. In addition to the duck, the tumor has been transplanted into pheasants, turkeys, pigeons, and guinea fowl [see Duran-Reynals (48) for references]. Virus variants have been obtained from these heterologous transfers as was achieved in the case of the duck. The degree of variation of the agent was inversely related to the ease with which the tumor was transferred into the species. Adult pheasants were susceptible to the agent, while pigeons were resistant.

The Rous sarcoma as well as other virus-induced tumors will grow in the anterior chamber of the guinea pig eye (121, 123). Following this sojourn in the rodent, there was evidence that the recovered virus was altered as seen by the incidence of periosteal lesions when inoculated into chicks. Serial passage from eye to eye in the guinea pig was successful for only two trans-

fers. The virus did not remain localized in the eye of the rodent since the agent was recovered from regional lymph nodes, spleen, and liver as early as 24 hr. after eye inoculation with the sarcoma and as late as the ninth day of tumor growth (124). In the few cases studied, the agent was not detectable in guinea pig fetuses. Transfer of the growth to the mouse eye is associated with no alteration of tissue specificity when reinoculated into chicks, and the agent was not detected in other tissues of the mouse in the cases studied (125). Greene (63) has found that when the Rous tumor is transferred subcutaneously into normal mice, only a small growth results. If, however, the mouse already possesses a spontaneous tumor or a transplanted fragment of embryonic tissue beside which the Rous tumor is placed, growth of the sarcoma is good. He has obtained best results by transfer of the chicken tumor to brains of mice, as well as by inoculation intracerebrally into guinea pigs and rabbits. In some instances growths may occupy a greater part of a hemisphere. These sarcomas have been carried serially for six passages in mice and guinea pigs. Also, mice inoculated intracerebrally with chick embryo tissue infiltrated with the Rous agent yielded typical Rous sarcoma after 20 days.

The cell of origin of the Rous sarcoma has been determined by two different techniques. Levine (79) making studies *in vivo* came to the conclusion that the fibroblast is the basic cell and that this is derived from monocytic cell types through elements he refers to as transitional. Tenenbaum & Doljanski concluded from tissue culture experiments that two cell types compose the Rous sarcoma. One of these is spindle in shape and the other is a basophilic round cell. Both are able to carry the agent, and cell environment determines which type predominates (136). Rous sarcoma has not been maintained in tissue culture over 50 passages without the addition of normal chick fibroblasts. It was possible to elicit tumors in birds at the 30th transfer, but beyond this the cultures lost vitality (137). The cause is not clear. This experiment might suggest that the agent of Rous sarcoma or some product thereof is responsible for the cancerization process, and also that the agent not only induced cell proliferation but also is ultimately toxic to these elements. It would be interesting to titrate the virus content of these cultures.

The Rous sarcoma is high in mucopolysaccharide which is similar if not identical to hyaluronic acid and in the opinion of Warren *et al.* is suitable for the assay of hyaluronidase (139). Burk *et al.* (22) have studied in detail the metabolic properties of the Rous sarcoma along with those of five other filtrable and chemically induced chicken tumors. In general, it was found that these neoplasms did not differ significantly from other malignant animal growths. Davidson & Waymouth (36) have also studied the chemical content of the Rous sarcoma and chemically induced fowl tumors.

It was pointed out by Roffo in 1926 that saline extracts of the Rous sarcoma when injected intravenously into chicks killed the birds promptly. Shrigley *et al.* (126) have studied this phenomenon and found that it is associated with intravascular clotting. Massive clots in the right heart and

great vessels are the usual finding. The effect can be prevented by heparin, the globulin trypsin inhibitor from soy beans, and the proteose trypsin inhibitor from navy beans. In our relatively purified celite eluents of the Rous tumor, the "toxicity" is equal to that of the original extract although the total nitrogen has been reduced to 10 to 20 γ per ml. This fraction also possesses powerful thromboplastic properties as determined by its ability to clot chicken plasma. It also has potent tumor-producing qualities and is not "toxic" to mice, rabbits, or guinea pigs. There is no association of this property of eluents with tumor necrosis, trypsin activity of the growth, nor with percolate or developer fractions of the pancreatic or small intestine chromatographic preparations which are high in trypsin activity. Spontaneous mammary gland carcinoma of a mouse was "toxic" for the mouse but not the chick, and while celite eluents, unlike those from the Rous tumor, clotted mouse plasma, it did not clot chicken plasma.

As a result of the observations of Duran-Reynals (45) that the Rous agent in young susceptible hosts produces few or no tumors but may be manifest by the hemorrhagic diseases described above, some interesting facts have come to light. While hemorrhagic disease is a manifestation of susceptibility, tumor formation is associated with partial resistance. This has been shown to be the case (46) since young susceptible chicks receiving adult chicken serum which contains antibodies against the agent develop more tumors than hemorrhagic disease. On the other hand, adult chickens may die of the Rous infection with lesions both hemorrhagic and neoplastic in type. Here it is believed that the bird's resistance is lowered in old age. As Duran-Reynals has pointed out, one can see an analogy between these observations and those involving human cancer.

Biochemical blood studies have been made on birds with the Rous tumor and on normal individuals by Dyer & Roe (55). While the examination of some 23 different blood constituents and properties revealed no significant differences, Caselli (29) felt from his studies that the plasma proteins were lowered in tumor-bearing birds.

As a result of his studies of the Rous virus infection in ducks, Duran-Reynals with King (47) tested the effect of natural antibodies of ducks and chickens against their respective species variant viruses. It was observed that duck serum would protect only against duck variant virus when inoculated into ducks. It was of no protective value when the variant was inoculated into chickens nor was it effective against chicken variant virus in chickens. On the other hand, normal adult chicken serum protected against both the duck and chicken variants of the virus regardless of which host was used. They interpret these findings to suggest that the duck virus has acquired specificities for the duck which the duck serum neutralizes; since these are not essential for infection of the chicken, the duck serum is unable to protect the chicken. The duck virus, however, also has chicken receptors which still function in the duck and therefore chicken serum protects both species against both agents.

Little *et al.* have found that folic acid is an essential constituent of the

diet of chickens for the development of the Rous tumor. Even though nicotinamide, calcium pantothenate, riboflavin, and cholic acid stimulated tumor response, folic acid was the only essential ingredient (80). Confirmation of this is seen in the fact that folic acid antagonists inhibit neoplastic growth (81). Vitamin B_{12} is also essential for Rous sarcoma proliferation. However, vitamin B_{12} and folic acid are not mutually exclusive, since when both substances are added to the diet the latent periods of the tumors are shorter and growths are larger (96).

One should not gain the impression from the above account that the Rous tumor is the only virus-induced avian growth. Duran-Reynals (49, 50) has described several and gives reference to others.

Before leaving the topic of chicken tumors it should be mentioned that attempts have been made to produce filtrable neoplasms in chickens by the injection of chemical carcinogens. McIntosh & Selbie (89) have reported success in this connection, but until recently their work has not been confirmed. Peacock (101) has critically reviewed their work and concluded that of all the growths they described, only one might fulfill the requirements of filtrability. Oberling & Guérin have obtained methylcholanthrene-induced tumors in legs of chickens (95). These growths subsequently showed metastases and were transplantable. In the fifth and sixth passages active filtrates were obtained through an L_2 Chamberland filter. The seventh passage tumor was not filtrable. Pieces of the growth kept in glycerine in the cold remained active after 69 days, and the chickens inoculated with the methylcholanthrene neoplasm were resistant to virus-induced growths but not to the virus of leukosis. Duran-Reynals (51) has found that in his experience, acute fowl pox develops in practically every bird painted with methylcholanthrene. If one continues to paint the bird with the carcinogen, chronic inflammatory reactions develop, as well as angiomas and discrete, proliferative, epithelial lesions. Upon inoculation of the bird with testosterone all of the lesions flare up and the epithelial growths become squamous-cell carcinomas. The virus of fowl pox can be demonstrated in these lesions for long periods of time, even until death in some cases. Duran-Reynals does not mean to imply at this stage of his experiments that fowl pox is the cause of the cancers so produced.

Avian Leukosis Complex

In general the avian leukoses may be divided into two groups, as recently discussed by Pikovski *et al.* (102). The first group is clinically characterized by the presence of lesions of the blood vascular system as well as sarcomas. These tumors often metastasize and may also be transferable. Cell-free leukotic material may induce these growths and on occasion they may appear in the absence of leukotic lesions. In the second group the picture is primarily leukotic, with tumors appearing rarely and not metastasizing or being capable of indefinite serial transfer. These strains vary greatly in their ability to elicit growths, from those yielding no tumors to those giving rise to many. One might speculate as to whether or not this complex represents a series of agents which are undergoing evolutionary change and eventually might

become stabilized as tumor-producing viruses. Perhaps the variation discussed above involving the tissue specificities of the Rous agent is a reflection of a similar evolutionary path. On the other hand, the possibility that the leukosis complex is the result of a mixture of viruses has not been excluded.

Pikovski & Doljanski (103) feel that in the second group the blood changes are virus-induced and that the tumors which develop result from the immature cells which are used in the transfer and which have potentialities of turning into fibroblasts. When leukemia results without growths, they consider the cells too mature to produce tumors. The authors feel that in the first group the agents have affinity for bone narrow cells as manifested by leukemia on intravenous inoculation, and for primitive fibroblasts as indicated by sarcomas on intramuscular injection.

Burmester (23) and his associates have also studied this complex extensively and have found that naturally occurring visceral lymphomatosis may be transferred by cell suspensions and in some cases by cell-free filtrates. Jungherr (72) has reviewed extensively the concepts of the avian leukosis complex.

Ultracentrifugates from filtered plasma of chickens infected with avian erythro-myeloblastic leukosis have been studied under the electron microscope by Beard *et al.* (9). They found spheroid particles with and without tails and evidence suggests that the latter had been torn from the spherical bodies in the process of purification. The round particles measured 60 to 100 mμ in diameter, while the tails were from 100 to 200 mμ in length. These particles had a sedimentation rate of 630S and were low in desoxyribonucleic acid. Since these bodies were not seen in the plasma of normal birds nor in that of chicks immune to avian leukosis, it is possible that the particles represent the etiological agent of erythro-myeloblastic leukosis. However, the authors indicate that more work is necessary before such a conclusion is tenable.

Spontaneous Mammary Gland Cancer in Mice

Studies of this growth are exceedingly extensive and have been reviewed up to 1945 in a monograph of the American Association for the Advancement of Science (92).

There are at least four factors which influence the development of spontaneous mammary gland cancer in mice. One of these is the genetic constitution of the host. The second is the hormonal makeup of the individual, which is of course a reflection of his genetic background. The third factor is the presence of a virus-like agent, the milk influence, which is transferred from mother to suckling offspring. This third factor is no less influenced by the hereditary status of the host than are the other two. The fourth includes the many influences of the external environment which are reflected in the mammary tumor incidence. To the microbiologist, these are not new relationships since all must be considered in any infectious disease. If one reflects for a moment upon the interdependence of these factors he can see the futility of championing any particular one as the cause of mammary cancer.

By way of environmental influences, Andervont (2) has shown that virgin females kept singly in separate cages developed more mammary cancer than those held in groups of eight. That this might be a result of different hormonal activities was suggested by the fact that the segregated females went into estrus earlier, remained longer, and had their cycles more often than did the grouped individuals. Diet as it affects mammary tumor incidence has been studied by Tannenbaum and his associates (135), by White *et al.* (141), and others. White and her collaborators found that the reduction of caloric intake by 50 per cent reduced spontaneous tumor incidence from 100 to 12 per cent in virgin C_3H mice. However, they also noticed that during this time the females were in anestrus. That something more than diet might be operating was indicated by the fact that C_3H animals on diets low in cystine, but into which were implanted subcutaneously pellets of diethylstilbestrol, yielded tumors in 45 per cent of the cases in spite of their state of inanition. Higgens & Woods (69) were able to restrict growth of transplantable mammary tumors in C_3H mice by the administration of the folic acid antagonist, aminopterin. This effect was not seen in mice on folic acid-aminopterin mixtures.

It is beyond the scope of this paper to consider in detail the genetic analysis of mammary gland carcinoma of mice. Bittner (12, 14) has recently reviewed the whole problem. Although complex, such an analysis is not much different from that for any infectious disease where clinical expression of an agent is dependent upon a susceptible genetic constitution. Results indicate that susceptibility is inherited on a multiple factor basis with a tendency toward dominance (13). When interpreting data from reciprocal crosses between high and low cancer lines, the observations of Andervont & Dunn (2a), as well as those of Mühlbock (93) that sperm from the epididymis of mice of high tumor lines may possess the milk agent, must be kept in mind. Also one must consider in the genetic analysis of this disease that the milk influence itself may possess potentialities for qualitative as well as quantitative change. If this agent is to be considered a true virus, it must be recognized that such macromolecular entities may be capable of genetic alteration, as pointed out by Luria (87).

A detailed consideration of the endocrinological aspect of the mammary gland tumor would also take us too far afield. Enough has been said to indicate the great significance of hormones in preparing the proper substrate upon which the milk influence may act. Parenthetically it should be pointed out that spontaneous mammary tumors do occur in mice without the milk influence (68). Bittner has expressed the thought (14) that the agent might alter the metabolism of the host in such a way that "carcinogenic" hormones may result. However, the milk influence in d and A strain mice appears not to alter the production nor action of estrogenic hormone, according to the report of Mühlbock (94).

Since its discovery in 1936, the so-called milk influence has been studied intensively. Barnum & Huseby (7) have recently reviewed the properties of this agent. The mammary tumor inciter is filtrable through bacterial filters,

sedimented by a force of 23,000 $\times g$ for 90 min., and resists glycerin as well as lyophilization.

The lactating mammary gland as well as spontaneous mammary carcinoma represent the best source of the agent. It is also present in the lung, spleen kidney, heart, and in small amounts in the liver. Whole blood is a relatively poor source of the agent (39). Bioassay of these tissues is made difficult by the long incubation period of the agent. It is interesting that while the milk influence is antigenic in rats and rabbits, eliciting neutralizing antibodies following the injection of relatively purified preparations, no such antibodies have been satisfactorily demonstrated in the mouse carrying the agent (61). By high-speed centrifugation, Barnum & Huseby (7) have obtained from lactating mammary glands of a high tumor line of mice a lipoprotein complex almost devoid of pentosenucleic acid. This fraction possessed tumor-producing potentialities which are equal to the original pentosenucleic acid-containing microsome preparation of the lactating gland. Aliquots containing 2.3 μg of total nitrogen per ml. have yielded a little less than 50 per cent tumors in susceptible mice. Similar preparations from lactating mammary glands of mice without the milk influence yielded fractions showing no significant differences from the above except in biological activity.

Electron microscope studies of relatively purified fractions of mouse milk and tissues have led to some confusion. Graff *et al.* (62) have obtained particles, from milk of mice from a high tumor line, which by electron microscopy averaged 98.5 mμ in diameter. These particles were not seen in milk from low tumor line individuals. However, when these mice lacking the milk influence were nursed by mothers who possessed it, the authors isolated particles which now averaged 72.7 mμ in diameter from the milk of the lactating females so nursed. While the investigators feel that the particles are associated with carcinogenesis, their bioassays to prove that they are exclusively so are still in progress. Passey *et al.* (99) have obtained, from normal and maligant tissues of three strains of mice with high tumor incidence particles which, as determined by electron microscopy, range from 20 to 120 mμ with an average diameter of 30 mμ. There are fewer of these particles in normal lactating gland than in mammary tumor extracts, and C_3H females possessing breast tumors induced by estrone, and combined estrone and methylcholanthrene, possessed more particles of this nature than did males of the same strain. Low tumor strains showed only a few of these 30 mμ particles. While the particles described by Graff *et al.* were susceptible to trypsin action, those of Passey *et al.* were not. Passey's bioassay experiments likewise are not complete, but so far 71 of 221 mice receiving material with particles have developed tumors, while among those who received fractions in which particles were not seen only 7 of 135 have tumors. Supernatant fluids "apparently free from particles" have to date not elicited tumors in susceptible mice (100). Porter & Thompson (104) have recently found spherical particles in the epithelial cells of spontaneous and transplanted mouse mammary tumor grown in tissue culture. These particles show a dense center averaging 75 mμ in diameter and an over-all diameter of 130 mμ.

One can see from the above discussion that results of further study must be awaited before any definite conclusion regarding the interpretation of these results can be made.

Bonser (16) feels that the milk influence acts mainly on the acini of the mammary gland, giving rise to a high degree of acinous proliferation which subsequently becomes intra- and extra-acinous carcinoma. In mice not having the milk influence but receiving estrogens, acinous proliferation develops but, with the doses of hormone used, the process stops at this state. She feels that the sequence of events leading to mammary gland carcinoma are: (*a*) acinous proliferation, (*b*) intra-acinous carcinoma, accompanied in some cases by intra-duct carcinoma, (*c*) extra-acinous or infiltrating carcinoma. In two high tumor lines, the fullest extent of acinous hyperplasia was seen in the presence of the milk agent. Acinous hyperplasia seems to be necessary as a preliminary stage before the initiation of cancer. It is not possible to discern the point of change to malignancy, nor which cells are first to make this change. Once the intra-acinous carcinoma is formed, one can not tell how long it will remain localized. Huseby & Bittner (71) have made comparative studies on the morphology of mammary glands from high and low tumor mice in the light of their respective capacities to develop cancer.

Infectious Myxoma and Fibroma of Rabbits

Infectious myxoma of rabbits is produced by a virus which possesses the ability to cause necrotizing as well as proliferative lesions. Ahlström (1) has given a detailed account of the histopathology of this disease. In general, the agent of myxoma, like that of fibroma, attacks undifferentiated mesenchymal cells. If the myxoma virus is low in virulence or its host relatively high in resistance, lesions are more proliferative and less necrotizing.

Smith & Kun (133) have studied the effect of the myxoma virus on the chorioallantoic membranes of fertile eggs and report the presence of brilliant acidophilic inclusions in ectodermal cells during rapid virus multiplication. Later, cells of all three layers of the membrane proliferate, the inclusions decreasing in number and becoming basophilic. Finally the process subsides with an eventual return to normal membrane. These workers (78) have made biochemical studies of infected egg tissues and observed an increase, in contrast to normal membranes, in aerobic lactic acid formation from glucose. Since increase in virus titer does not run parallel with this glycolysis, the authors feel that the agent does not possess glycolytic activity. The fibroma virus has likewise been grown on the chorioallantoic membrane (134). Balls *et al.* (6) were able to sediment the active agent of myxoma at 34,000 $\times g$ for 2 hr., and found that biological activity was associated with the presence of a steroid, which was not cholesterol, and was not present in normal rabbit tissue. Whether this lipid is a constitutent of the virus or a degradation product of the diseased cells is not known.

That the virus of myxoma and of fibroma are antigenically related has been established. Shaffer (118) has studied virus suspensions of myxoma and fibroma and has concluded that each contains at least two distinct comple-

ment fixing antigens. One of these is present only in unfiltered viral suspensions and is heat-stable (98°–99°C), while the other, heat-labile, is present in filtered material. Again, whether the components represent the virus or the effect of the agent on the tissue is not clear. The experiments of Berry and Dedrick, later extended by Berry (10), are of great interest in this connection. By mixing heat-killed myxoma virus with living fibroma agent, it was possible to transform the fibroma virus into that of myxoma. In the light of Avery's work on the transforming substance of the pneumococcus, this observation is particularly intriguing [see McCarty *et al.* (88)].

The relationship between virulence of the fibroma agent and host resistance has been studied by Duran-Reynals (52). Following inoculation of newborn rabbits with the fibroma virus, these animals yielded agent from blood and viscera, contrary to experience in adult infection. If large enough doses were used, the newborn rabbits died of an acute inflammatory disease which was not unlike myxoma in adult animals. When newborn rabbits received only small quantities of virus the reaction was similar to that in adult animals Andrewes & Shope (3) have described variations in the fibroma virus that give a more intense inflammatory reaction in adult rabbits than the unaltered agent. This, therefore, is another example of a virus which is accepted as one initiating cell proliferation, yet in hosts of low resistance yields acute destructive lesions (53, 54).

Papillomas of the Rabbit

It is well known that the papillomas described by Shope in wild cottontails are virus induced. While the agent will elicit benign growths in domestic rabbits, some difficulty is associated with its recovery from these lesions. Serial passages of growths in domestic rabbits have been carried out for 14 transfers by Shope and for 12 by Selbie & Robinson. The latter investigators, collaborating with Shope (117), transferred the virus which had been used serially in domestic rabbits to cottontails with the idea of increasing its vigor. However, this effect was not realized, since adaptation for the domestic rabbit was lost by this one transfer to the cottontail. Friedewald has increased the susceptibility of the domestic rabbit's skin to the agent by rendering the surface hyperplastic and protecting the inoculated area with paraffined gauze until healed (59).

It has long been recognized that these benign papillomatous growths may become malignant, the incidence being higher in domestic rabbits than in wild cottontails, an observation in agreement with Syverton *et al.* (129). Associated with this change is an inability to demonstrate the presence of the papilloma virus, yet it has been considered to be present because of the continual elaboration of antibodies against it by the rabbit host. The growth of the benign lesions may be divided into three phases (129); the proliferative, the stationary, and the involutionary phase. When malignancy takes place in the involutionary phase, papilloma cells are replaced by anaplastic elements. The critical stage for malignant change is at the ninth month of papilloma growth, both for the cottontail and the domestic rabbit (129).

Papillomas were rarely found to continue after 18 months. In another study, the same authors found that the incidence of malignant change of papillomatous growths was no different in the domestic than in the cottontail rabbits, a circumstance which they feel more accuately represents the facts (130). However these two hosts varied in other respects in their reaction to the Shope agent. The virus was isolated from benign lesions in cottontails up to the onset of the involutionary phase or the development of malignancy. Further, contrary to the observations in domestic rabbits, when papillomas were removed by trauma or x-ray from the cottontail the lesions would return.

The interpretation of the association between virus and malignant growth derived from the papilloma is not clear. However, the studies of Kidd (73) have led him to believe that the cancer cell and virus hold something other than a casual relationship. On the other hand, Syverton *et al.* (131) feel that the papilloma agent is "limited to that of a provocative carcinogenic agent and not to that of an activating carcinogen," and they doubt that the agent plays an essential part in the ultimate formation of malignancy. Their conclusion is based upon the fact that in their experience carcinomas did not elicit papillomas or antibodies for the Shope papilloma virus when transferred from the original Western hosts to Eastern cottontails.

In a recent review (74) Kidd points out than an epidermoid carcinoma, V_2, which developed from a Shope papilloma 12 years ago, is now in its sixtieth odd transfer in domestic rabbits. Some time between the 22nd passage and the 46th it apparently lost the papilloma virus, since it no longer elicits antibodies against the agent. One might conclude from this that the agent is not essential for the maintenance of the V_2 carcinoma in its present state. A detailed study of the macromolecular constituents of the V_2 carcinoma (75) has revealed a specific antigenic substance in the cancer which fixes complement. This antigen is not found in normal rabbit tissue nor in the benign papillomatous growth. The Brown-Pearce tumor of rabbits, which is another malignant growth of unknown etiology, likewise possesses a specific complement-fixing antigen, but this is distinct from that of the V_2 tumor. Neither of these antigens has the ability to initiate the growth of their respective tumors. Unlike the antibodies against the specific component of the V_2 tumor, antibodies against the Brown-Pearce specific component inhibit the growth of the latter neoplasm.

A second carcinoma (V_7), derived from a Shope papilloma, is now being studied (107). This new growth, although histologically distinct, contains an immunologically active component which appears to be antigenically identical with that of the V_2 tumor. The V_7 carcinoma readily develops in suckling rabbits but rarely elicits antibodies against the papilloma agent or the specific complement-fixing component. In 50 per cent of the animals it has been possible to recover the papilloma virus in small quantities from three cancers, including the V_7, grown in newborn rabbits. Recovery of the agent from growths of the 3rd and 4th transfer was not successful.

Studies of the properties of the Shope papilloma virus show that it is exceedingly stable, remaining active in 50 per cent glycerin at 4°C. for 20 years (57). The size of the dehydrated particle is in the neighborhood of 65 mμ (119), and consists of a nucleoprotein of the desoxyribose type, being quite low in lipid (8). Knight (77) has found 18 different amino acids present in the purified virus material. None seems to be found in a particularly large quantity, and similar to the findings in other viruses, these amino acids are mostly acidic in type. The Shope agent is exceedingly resistant to x-ray; Syverton *et al.* used 14 million r to destroy it (132).

Metabolic studies of the V_2 carcinoma by Kidd *et al.* (76) indicated that it possessed no unusual properties not present in other malignant growths. Also, papillomas yielded biochemical findings similar to other benign tumors which were found not to vary too extensively from results with normal skin.

Rous and his associates (60) have studied in detail the action of carcinogens on the rabbits' skin. Particularly pertinent to our present discussion is their observation in domestic rabbits that virus-induced papillomas to which methylcholanthrene or tar has been applied, become malignant more rapidly than under natural conditions (110). Previous attempts have been unsuccessful, in spite of the presence of extensive hyperplasia, to shorten significantly the period between onset of the benign growth and the beginning of cancer. In the above report, however, the malignant changes in the growths were not associated with an ordinary preliminary stimulation of cell proliferation, but appeared to be the result of the carcinogen acting on the papilloma cells in their natural state. In a recent study (111), a similar result was obtained when domestic rabbits, whose skin surfaces were rendered hyperplastic by turpentine, were treated with methylcholanthrene or 9,10-dimethyl-1,2 benzanthracene-infiltrated bandages. Also, some areas were inoculated with the Shope virus and likewise covered by these carcinogen impregnated dressings. Those portions of the skin receiving only the hydrocarbons yielded no growths, while the virus-infected control areas developed the customary papillomas. However, in the areas receiving virus plus hydrocarbon, malignant tumors soon developed. It is interesting to note that three to five months after the application of the hydrocarbons to the skin, chloroform, a noncarcinogen, yielded in these previously 9,10-dimethyl-1,2 benzanthracene-treated areas which were devoid of growths, papillomas morphologically distinct from the virus lesions.

If papilloma virus is injected intravenously into rabbits already possessing tar-induced growths, the agent frequently localizes in them with the result that some become morphologically similar to virus-induced papillomas, some do not make this change completely, and others do not convert at all but rapidly increase in size. On the other hand, some of the benign lesions undergo malignant change [see (110) for references].

Parsons & Kidd (98) studied rabbit oral papillomas which proved to be entirely unrelated to the growths described by Shope. While virus-induced, they remain benign, although their size is increased by the presence of tar.

Papillomas in Other Animals and Man

Infectious oral papillomatosis has been reported in dogs by DeMonbreun & Goodpasture (37). These growths, always benign, are produced by an agent which is quite specific for tissue and species. The oral mucosa is the only location of the lesions, and all of the usual laboratory animals including kittens and monkeys are resitant to the agent. These growths eventually regress, leaving the host immune to further infection.

Cattle likewise become infected with a virus which produces warts (56). The lesions may appear on the muzzle, back, belly, or udder, and the infection may be transferred naturally from one animal to another by contact, or experimentally by filtrates.

Recently, Lucké *et al.* (86) have described a spontaneous, transplantable papilloma first observed on the brow of a Cebus monkey. Transfer is achieved by rubbing into scarified areas cell suspensions of frozen or glycerinated material. New World and Old World monkeys alike may be infected, and there is no evidence of malignancy, the lesions regressing in 4 to 6 months.

In man, the common warts have been shown to be virus-induced. The clinical appearance of these lesions frequently depends upon their location on the body (56). Recently, Strauss *et al.* (128) have studied extracts of common and plantar warts under the electron microscope. These materials have yielded spherical particles arranged in crystalline-like clusters. The particles were 50 to 53 mμ in diameter when in clusters, and a little larger when occurring singly. Under the ordinary microscope intranuclear inclusions were also observed in tissue sections, as well as cytoplasmic granules which developed and finally fused into larger masses as the cells matured. Blank *et al.* (15) have studied these intranuclear inclusions in detail and have concluded, contrary to Strauss *et al.*, that they are nucleoproteins of the desoxyribose type. As found by Strauss *et al.*, however, these inclusions were not seen in all verrucae.

Before leaving this section, it might be well to call attention to recent interest in the use of the electron microscope in studying human cancer extracts. Several workers have described virus-like particles in these extracts and some have suggested that these might be evidence for the virus etiology of the growth. It seems that a considerable amount of caution should be exercised in interpreting these findings. One is reminded of the enthusiasm of a few years ago when many investigators reported the presence, in tumor cells and tissues of cancer patients, of inclusion bodies suggesting the existence of a virus, Recently, Fox (58) has studied by electon microscopy virus-like bodies from human tumors and has used the filtrates of the Rous sarcoma as a "control" to aid in their identification. This is rather an unfortunate standard for comparison since we (122) have found a great variety of "virus-like" particles in relatively purified celite eluents of the Rous tumor and are not yet sure which might represent the agent.

Infectious Pulmonary Adenomatosis of Sheep

Jaagsiekte of sheep has been described in detail by Dungal (40). The pathologic changes are strictly limited to the lungs which show small grayish

spots tending to become confluent. Cellular exudate in the alveoli may be pronounced. The pulmonary epithelium takes on the character of a benign adenoma and occasionally appears malignant. Experimental attempts to transfer the disease have met with some difficulty. The most successful method was to allow lambs to breathe the exhaled air of an infected animal. While tranfer by filtrates was possible, better results were obtained if inoculations were accompanied by intratracheal instillations of "bacillary cultures causing pneumonia in sheep." The presence of lung worms in the prospective host facilitated the transfer of the disease by filtrate. The author, however, feels that the lung worm does not carry the virus.

While this disease is similar to one reported occasionally in man, no interrelationship is know. Dungal states that sheep herders who have worked intimately with infected animals have shown no signs of the disease

The Virus Etiology of Cancer

Gye and his associates (66) have reported that in mice, spontaneous and induced sarcomas which have been dried, minced, and frozen at −79°C. retain their ability to produce growths in their original host strains, and also in mice of four different genetic lines. As the result of extensive studies these workers are convinced that intact cells as such cannot survive such treatment, and suggest that a virus may be responsible for the development of these growths. The evaluation of this conclusion of course depends upon the validity of the statement that all intact cells are killed by the processing. Not all investigators agree that this is the case. Hirschberg & Rusch (70) have recently reviewed this subject, and Warner & Gostling (138) present the background of this approach.

The possibility that a virus may be the cause of cancer has been discussed by several authorities in the field (4, 35, 41, 67, 109) but obviously a detailed consideration of the problem cannot be undertaken here. However, as a result of our present discussion there are certain points which might be brought out. It seems likely that viruses may stimulate cells to proliferate even to the extent of producing benign growths. Whether they are essential for change to malignancy is a question that is not yet clearly answered. True, there are malignant growths which are associated with viruses, but do we know that these malignant tumors have not at first had benign phases which have given way to malignancy while still undetected?

Enough analogies have been drawn between cancer and the behavior of known infectious processes caused by viruses to make one realize that there is a close similarity between the two. It is also well appreciated that the known properties of viruses may fit the theoretical requirements of an etiological agent for the cancerous process. It would now seem more profitable to leave this approach to the problem, to review the known facts regarding certain growths, and to determine if there exists evidence for a thread of consistency which might follow through tumors of known and unknown etiologies. The findings of Kidd that carcinomas derived from virus-induced tumors possess specific antigenic substances, and his observation that the Brown-Pearce tumor likewise possesses an immunologically specific

component, are significant. Particularly important is the fact that antibodies against this Brown-Pearce antigen inhibit the growth of the tumor. There has been a considerable amount of careful work done by Maculla (90) to detect specific antigenic components in malignant tumors of the mouse. She has found that nucleoproteins derived from some mouse growths are distinct from those derived from normal organs.

It is interesting to speculate upon the origin of these specific components. One wonders if they may perhaps represent remnants of a tumor-producing virus which, due to an altered physiological environment as a result of an aging growth, has lost its ability to elicit tumors yet still maintains immunological specificity and its capacity to reproduce. That viruses of inflammatory diseases may undergo such changes is well recognized. Schlesinger (114) has observed that in the mouse brain it is possible to get an increase in the complement-fixing antigen of the influenza virus as well as an increase of the hemagglutination titer without an associated rise in infective titer of the virus. This newly formed "incomplete" virus is unable to infect new hosts, but is antigenically similar to the original agent. Recently, Salk & Bennett (112) have obtained results with the Lansing strain of poliomyelitis virus which suggest that passage in the monkey is associated with a partial loss of pathogenicity for mice, yet its ability to elicit protective antibodies in mice is as great if not greater than in the mouse-adapted strain.

Is it possible that the growths which we have been considering represent an evolutionary series from tumors of known etiology to those in which the cause is not known? Do these macromolecular, specific substances represent all that is left of an agent that at one time initiated the benign growth from which the present cancer arose? Is it possible that each tumor we have discussed represents a different stage in this spectrum? The cancers lacking evidence for any specific component may have evolved sufficiently to lose all trace of their exciting stimulus. The tumors like the V_2 carcinoma in which antibodies against the specific component will not inhibit growth might represent conditions in which the original agent has been more modified than, for example, in the case of the Brown-Pearce tumor where antibodies against the specific antigen inhibit the growth. It would be most interesting to see whether other virus-induced tumors, such as the Rous sarcoma, might have specific antigens which will elicit antibodies that inhibit the tumors yet be distinct from the agents. It would also be significant to alter the virus experimentally and obtain specific antigens with the power to infect but not elicit growths, yet whose antibodies would prevent normal virus activity. There is much important and fascinating work still to be done in this field.

The possibility that these immunologically specific components of tumors might be the result of a gene mutation within the growth itself is suggested by the recent report of Levine (79a). This investigator found in a patient suffering from a carcinoma of the stomach, lytic antibodies against the red blood cells of some 1,400 other persons tested. The cells of the patient and her sister, together with one sample of blood from South Africa, were the

only ones not lysed. Carcinoma suspensions from the patient specifically absorbed these antibodies from the individual's serum, suggesting that the tumor was the source of the antigen. The lack of the antigen on the patient's and her sister's red cells suggests that the gene responsible for this characteristic was not received from either of their parents, since antigens of this type have been shown to be inherited. There was no history of transfusion nor antibody stimulation from previous pregnancies, and the origin of the tumor antigen from contaminating bacteria seemed unlikely. The evidence therefore suggests that this immunologically specific tumor component was endogenous in its origin and might have resulted from a somatic mutation within the tumor tissue. The possibility that the patient and her sister might represent exceptions to the rule in not having the antigen on their red blood cells yet possessing it in their normal tissues is being investigated.

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PHYSICAL AND CHEMICAL CHARACTERISTICS OF VIRUSES[1]

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One of the principal problems of virology remains that of the relationship of this group of infectious agents to their microbiological analogues of the plant and animal kingdoms. Due to the lack of suitable biological criteria, knowledge of the chemical and physical characters of viruses is, at the present time, the major basis for the formulation of concepts of the nature of these agents [Pirie (74)]. With the demonstration by Schlesinger (90), Craigie & Wishart (24), and Stanley (106) in the early 1930's that viruses could be purified, there was established a pattern [Wyckoff (134)] for the biophysical and biochemical examination of the individual agents. In this most productive era, investigation of representative viruses of all categories of hosts has established a new field of virus research and an extensive knowledge of the constitution and physical structure of viruses [Stanley (108), Hoagland (43), Pirie (73), Beard (11), Bawden (6), Knight (50), and Steinhaus (111)].

Constitution.—Viruses are remarkably complex and varied in composition (6, 11, 43, 50, 73, 108), despite smallness of size. All viruses contain protein and nucleic acid which, in various proportions, are the sole known components of the plant (6) and insect [Bergold (13), Wyatt (132, 133)] viruses. The amount of nucleic acid of the pentose type in plant viruses, with few exceptions, is not large (6); Stanley (109) found about 40 per cent in the virus of tobacco ring spot and Markham, Mathews & Smith (61) 28 per cent in that of turnip yellow mosaic. In the polyhedral viruses of the silkworm and the gypsy-moth caterpillar, Bergold (13) found only desoxypentosenucleic acid which, in the latter, Wyatt (133) estimated as about 15 per cent. Neither plant nor insect viruses contain lipid or carbohydrate in excess of that present in the nucleic acid.

The bacterial and animal viruses, particularly the latter, are more complex. The various bacteriophages are remarkably similar in constitution [Schlesinger (90), Northrop (71), Taylor (118), Cohen & Arbogast (22), Kozloff & Putnam (52), Csáky *et al.* (25)]; they all contain much nucleic acid (about 40 per cent) principally, if not all (21, 22, 25, 52), of the desoxypentose type. Lipid, likewise, appears to be a component of bacteriophages (25, 52, 90, 118). Variation in the constitution of the animal viruses is extreme. The range of nucleic acid content is relatively narrow, about 5 per cent in vaccinia [Smadel & Hoagland (102)] to nearly 10 per cent [Taylor (119)] in the papilloma virus. The nucleic acid is wholly of the desoxypentose type (102, 119). Taylor *et al.* (121) found only the pentose type in equine

[1] The survey of literature pertaining to this review was completed in January, 1951.

encephalomyelitis virus. In the influenza viruses, Knight (47) has reported both kinds. Taylor *et al.* (119) found that the papilloma virus appears to lack a lipid component; larger amounts were found in the vaccinia [Hoagland *et al.* (44)] and influenza [Taylor (117)] viruses, while approximately half (121) of the equine encephalomyelitis virus is lipid. Only the influenza virus contains carbohydrate [Taylor (117), Knight (47)] in excess of that found in nucleic acid.

Analyses of amino acid content of plant [Knight (49)], bacterial [Polson & Wyckoff (75)], insect [Wellington (128)], and animal [Knight (48)] viruses have demonstrated not only a wide range in the content and distribution of these constituents from one agent to another, but significant variations between types and strains of the same virus. The differences and variations in constitution among viruses are seen likewise between viruses and their host cells. Taylor (118), Wyatt (132), Kozloff & Putnam (52), and Csáky *et al.* (25) have shown differences in nucleic acid, lipid and carbohydrate of one or all of the T_2, T_5, T_6, and T_7 bacteriophages and their host *E. coli*. Such differences extend to the distribution of the bases of desoxypentose nucleic acids (132). Wellington (128) has found tryptophan absent from a polyhedral and a capsular insect virus but present in the respective polyhedra and capsules.

Size.—As in 1942, when Markham, Smith & Lea (62) discussed the subject, the full size range of viruses remains still to be determined, though the status of the problem has been immensely improved by electron micrography. Few viruses are of uniform size, and extreme variation in particle size is characteristic of some viruses. Consequently, excessive efforts at exact measurement of size are of limited purpose, and calculation of "molecular weights" is superfluous, to say the least. A valuable contribution of electron micrography has been the observation that viruses may not be as small as once thought. Values obtained by the methods of ultra-filtration developed by Elford (31) have indicated a range (62) of 2 to 5 mμ for some of the bacteriophages to 300 mμ or greater for the largest agents. Bodies at the low end of the scale have been regarded as too small to contain the complex structure associated with processes vital to life and reproduction, a view strengthening concepts of viruses as molecules. However, the smallest spherical virus to be purified, the Rothamsted strain of tobacco necrosis virus of Bawden & Pirie (7), is not less (6) than 17 mμ in diameter. Some of the smallest sizes are widths of relatively very long rods. Other very small values, such as 3 to 8 mμ reported by Levaditi and associates (58) for the colidysentery C-16 bacteriophage, were not substantiated by Giuntini *et al.* (35). It scarcely needs emphasis that there is a vast difference, eight-fold, between the content of a particle of 20 mμ diameter and one no smaller than 10 mμ. Even so, however, Hall (39) has demonstrated considerable complexities in protein molecules of 8 mμ diameter. Thus, there is no chemical or physical basis in either theory or fact for the supposition that any viruses are too small to support the degree of organization required for living matter.

Morphology.—Viruses affect many plant and animal hosts, but the recog-

nized distribution is greatly restricted. Only the Angiosperms, of the Spermatophyta, are known to be parasitized (6); parasitism of a second plant phylum by viruses, the bacteriophages, is likewise limited, seeming to involve only two orders, Eubacteriales and Actinomycetales, of the Thallophyta. Among the animals, a limited number of groups of the insects are affected [Steinhaus (112)], and of the vertebrates, the principal recognized hosts are the mammals and the birds.

The morphology and structure of viruses are of a range and complexity unexpected of such small beings; yet the various forms are old and familiar ones to the biologist, and in particular to the bacteriologist: (*a*) the coccoid types, spheres, spheroids and biscuit forms or very short rods; (*b*) bacilliform types, rods of ordinary proportions, filaments or long, thin rods; and (*c*) the sperm or tadpole shapes which are modified spheroids with tails or modified bacilliform bodies with head structures. The morphology of a given virus is related, to some extent, to the kind of host. The plant viruses, with one known exception, are spheres or long rods. The bacterial viruses are spheres or tadpole-shaped entities, while the animal viruses vary from spheres to sperm shapes. The insect viruses, with a possible exception, are bacilliform; no tadpole or sperm-shaped form affects either the insects or plants, and no bacilliform virus parasitizes either the mammals or the birds.

Plant virus spheres show relatively little species variation in size, ranging from 17 mμ (6) [Rothamsted culture of tobacco necrosis virus (7)] to about 39 mμ in diameter [tobacco leaf curl virus as found by Sharp & Wolf (101)]. Plant virus spheres of a given species are highly uniform in size and shape, and some form beautiful three-dimensional crystals. Wyckoff and his associates have obtained elegant electron micrographs of crystalline tobacco necrosis (63, 64) and Southern bean mosaic (77) viruses. These are the smallest, 17 mμ, and the largest, 26 mμ, virus spheres observed in unequivocally crystalline form. These viruses approach but are still of a different order of size from some crystalline proteins, such as the 8 mμ spheres of edestin shown by Hall (38).

The narrow and comparatively long plant virus rods, too, exhibit relatively little species variation. From the estimations of Bawden *et al.* (8), Stanley & Anderson (110), Williams & Wyckoff (130) and Knight & Oster (51), tobacco mosaic virus, for example, is about 15 mμ wide and 280 mμ in length. Some of the cucumber viruses measured by Knight & Oster (51) may be still narrower, 13 mμ wide, and 700 mμ long. A bacilliform plant virus is the agent of potato yellow dwarf disease, estimated by Black *et al.* (17) as about 50 mμ thick and 200 mμ in length, proportions like those of the bacilliform viruses of the insect diseases. Some of the rod viruses, under proper conditions, form paracrystals [Stanley (107)] which exist in two dimensions as shown by Bernal & Fankuchen (15) and Wyckoff (135). Stahmann and his associates (105) have relieved the monotony of this sphere and rod morphology with electron micrographs of the Wisconsin pea-streak virus which occurs as long, curved, interlacing threads 10 to 14 mμ in width and up to 5,000 mμ in length. Due possibly to small size, no

definite evidence of internal structure has been observed in either the spherical or the rod-shaped plant viruses.

Among the bacteriophages there again is relatively little variation in the parasites of different forms of bacilli [Kerby *et al.* (46), Hook *et al.* (45), Wyckoff (137), Baylor *et al.* (9)], cocci [Parmelee *et al.* (72), Luria *et al.* (60)], and fungi [Reilly *et al.* (83) and Woodruff *et al.* (131)]. The spherical or spheroidal bacteriophages vary from about 45 to 73 mμ diameter [(46), Baylor *et al.* (9), Anderson (1), Delbrück (29)]. The T_7 phage (46) not coated with metal [Williams & Wyckoff (129)] is of low contrast with indefinite periphery but contains an irregular region of internal structure marked by higher electron-absorbing power than the remainder of the virus. Crystals of these spherical entities are not seen despite high uniformity of particle size.

The different tadpole or sperm-shaped [Ruska (84, Hook *et al.* (45), Luria & Anderson (59), Putnam *et al.* (79), Woodruff *et al.* (131)] bacteriophages have heads which are short rods with conical ends or spheroids (59). The tail piece may be relatively broad (45), 18 mμ wide and 118 mμ long with a rigid, blunt or brush-shaped termination, or it may be slender, tapering and curved rather than rigid (59). In electrophoresis and diffusion experiments, in saline solution at low temperature, neither Sharp (92) nor Putnam *et al.* (78, 79) saw any evidence that tailed phages are mobile.

The head pieces of the tailed T_2 and T_6 agents, coated with metal, cast long shadows indicative of rigidity and little shrinkage in the coating chamber. Micrographs obtained (45, 79) without shadowing show but an indefinite rod shape enclosing a bow-tie shaped inner material. This structure has the appearance of segregated nuclear material, a probability in accord with the large amount of nucleic acid of bacteriophages which would be expected to be of relatively high electron-absorbing power. In some conventional micrographs there are "ghosts" (45) showing only the tail pieces and the outline of the head, as if liquid internal contents had escaped a durable and rigid envelope.

The recent pioneering work of Bergold (12, 13) has revealed unequivocally, for the first time, the character of some of the insect viruses, as summarized by Steinhaus (111, 112). Those clearly demonstrated in micrographs are rod-shaped and notably uniform in size, about 30 to 50 mμ in width by 250 to 350 mμ in length. Smith & Wyckoff (104) regard some as spherical.

The polyhedral viruses of the silkworm, the nun-moth and gypsy-moth caterpillars [Bergold (12, 13)], the yellow-striped armyworm, alfalfa caterpillar, and California oakworm [Steinhaus (111)], the tent caterpillar [Steinhaus *et al.* (113)], and the spruce bud worm [Steinhaus & Thompson (114)], are contained within the inclusion bodies and are liberated by solution of the polyhedral body in dilute Na_2CO_3. The capsular diseases of the fir-shoot roller (13), the variegated cutworm (113), the buckeye caterpillar (114) and the salt-marsh caterpillar (114) are characterized (13) by the occurrence of cytoplasmic or nuclear granules about 250 by 450 mμ in size.

A single bacilliform virus particle, about 40 by 300 mμ, is enclosed in each granule, which may be partially dissolved in dilute Na_2CO_3 solution, leaving behind the "ghost" shroud (114) about the virus.

Mammalian and avian viruses range from uniformly spherical particles to spheroids, to biscuit shapes and to the sperm shapes. Essentially spherical viruses are those of rabbit [Sharp *et al.* (94, 97), Neurath *et al.* (70), and Schachman (87)] and human [Melnick and associates (115)] papillomatosis, equine encephalomyelitis [Sharp *et al.* (95), Bang & Gey (5), and Taylor *et al.* (120)] and mouse encephalomyocarditis [Warren (125)]. Of these, the smallest is the encephalomyocarditis virus which is approximately 30 mμ diameter, while the rabbit papilloma virus appears, from sedimentation data [Sharp *et al.* (96)], to be the largest, 65.6 mμ. A still smaller particle, about 24 mμ in diameter, is believed by Warren (126) to be the Texas type of Coxsackie virus. All of these agents are highly uniform in size and shape. A round, centrally placed internal structure is clearly seen in the papilloma virus (94), surrounded by material fading inperceptibly into the background. The internal structure of the equine encephalomyelitis virus (95) is well defined, being of circular or lenticular shape, eccentrically placed in the virus particle, and it may be multiple.

Other bodies of spheroidal shape have been found associated with certain conditions, but their specificity remains to be established. Among these is the Coxsackie (125) virus. Porter & Thompson (76) saw apparently spherical particles in mammary carcinomas of the mouse, and Claude & Porter (20) reported analogous bodies in cells of Rous sarcoma.

Larger agents of different morphology, as seen by Sharp and associates and by Lauffer & Stanley, are the three types of influenza virus, human A (56, 122) and B (100) and swine (123), and the virus of avian infectious bronchitis pictured by Reagan *et al.* (81). The influenza viruses measured by Sharp *et al.* (98, 99) and by Lauffer & Stanley (56), are all about 100 mμ in diameter. They are spheroidal or bean-shaped and, moreover, vary greatly in size (56, 98) in a given preparation. On treatment with formaldehyde the particles seem to "ball-up" [McLean *et al.* (66)] but still vary in size. In conventional micrographs (100, 122, 123) the periphery of the particles is well defined, and there is a definite region of internal structure. In the shadow micrographs of Williams & Wyckoff (129) the particles show smooth surfaces and little evidence of distortion.

Further along in the scale of size, and in departure from the spherical, well defined and relatively rigid particles, are the viruses of mumps [Weil *et al.* (127), Elford *et al.* (32)], varicella [Ruska (85), Nagler & Rake (69), Rake *et al.* (80), Farrant & O'Connor (34)], herpes zoster [Ruska (85), Rake *et al.* (80), Farrant & O'Connor (34) and Evans & Melnick (33)], herpes simplex [Coriell *et al.* (23)], and fowl plague [Elford *et al.* (32) and Schäfer & Schramm (89)]. These viruses, 191 ± 31.6 mμ, 210×243 mμ, 196×218 mμ, about 200 mμ and 110 ± 16 mμ in size, respectively, are approximately spheroidal and vary greatly in size. In shadow micrographs they are essentially round but greatly flattened (32, 80, 127), as if, like amoebae or leuco-

cytes, the particles lack both internal and external supporting structures. The mumps virus shows (127) internal structure, and a notable characteristic is roughening (32, 127) of the external surface of the particles shadowed with metal.

A large group of viruses of similar morphology are those responsible for the pox disease of man or animals, including small pox [Nagler & Rake (69), van Rooyen & Rhodes (124)], vaccinia [Green *et al.* (36), Sharp *et al.* (97), Dawson & McFarlane (28)], fowl pox [Boswell (18), Groupé *et al.* (37)], canary pox [Ruska & Kausche (86), Groupé *et al.* (37)], myxoma of rabbits (86), mouse ectromelia and molluscum contagiosum (18, 86). Their sizes in electron micrographs are 244×302 mμ (69), 222×284 mμ (92), 264×322 mμ (18), 263×311 mμ (86), 233×287 mμ (86), 210×300 mμ (18), and 226×302 mμ (18), respectively. The particles of vaccinia have a somewhat rectangular form with rounded ends and, shadowed, appear low and flattened and are described as biscuit or brick-shaped (28, 97). The nuclear structure initially seen in conventional micrographs (36) is beautifully shown in the pictures obtained by Dawson & McFarlane (28, 65) with the virus treated with pepsin. Further evidence is seen in these pictures of the shape and size of the particles (three-dimensional size of 250 ×200 mμ×110 mμ thickness) and of the enveloping membrane.

Of characteristic morphology are the viruses of psittacosis, ornithosis, human pneumonitis, mouse pneumonitis, meningopneumonitis, lymphogranuloma inguinale [Kurotchkin *et al.* (54), Heinmets & Golub (42)], and feline pneumonitis [Kurotchkin *et al.* (54) and Hamre, Rake & Rake (40)]. These agents, the largest of the virus group, are about 400 to 450 mμ in diameter and vary greatly in size. The psittacosis virus pictured by McFarlane (65) and the feline pneumonitis virus in the micrographs of Hamre *et al.* (40), contain dense central material lying in a structureless semi-fluid substance enclosed in a rather strong membrane. The particles flatten and shrink on drying (40), looking like a wrinkled pea or a rubber ball pushed in on one side.

The exceptional morphology of the sperm-shaped virus of Newcastle disease of fowls [Bang (2, 3, 4), Cunha *et al.* (26), and Elford *et al.* (32)] remains controversial. In a balanced salt solution of physiological strength, there was seen by Cunha *et al.* (26, Fig. 1), in a limited number of micrographs, a profusion of exceedingly graceful-appearing sperm-shaped entities with heads slightly longer than broad to which were attached long, curved tapering tails two or three times as long as the heads. A few of such forms are shown in the micrographs by Bang (3). The occurrence of this form of Newcastle disease virus has recently been confirmed beautifully in micrographs of the agent from bat brain reported by Reagan, Smith & Brueckner (82). Other sorts of particles constitute the predominant content of most preparations, many of which are obviously fragments of the sperm forms while others may be stages in the reproductive process as mentioned below.

Certain spheroidal particles seen by Bang and by Elford and his coworkers in suspensions of low salt content were observed and described by

Cunha *et al.* as particles swollen and distorted by water taken up osmotically. Similar swollen bodies of the T_2 phage of *E. coli* under like conditions have already been described by Hook *et al.* (45). Green *et al.* (36) demonstrated like forms and limiting membranes of vaccinia virus, and the T_2 phage of *E. coli* (45) also has a limiting membrane. Excellent evidence of a semipermeable membrane was seen by Sharp *et al.* (99) with influenza and by Smadel, Pickels & Shedlovsky (103) with vaccinia viruses. The amorphous particles in chorio-allantoic fluid [Bang (3, Fig. 1)] are of dubious significance since such particles have been seen by Sharp *et al.* in the chorio-allantoic fluid of eggs injected with influenza virus [*cf.*, (100, Fig. 9A)]

Recently another form nearly identical with the Newcastle disease virus was isolated by Beard *et al.* (10) from the plasma of chickens with erythromyeloblastic leucosis. These particles varied from spheroids to sperm shapes with intermediate structures similar to those of Newcastle disease virus. The heads of the sperm-shaped particles were rather rounded, about 60 to 100 mμ in diameter, and the tails were about 200 mμ long, sizes much smaller than those of the agent of Newcastle disease (26).

Reproduction.—The mechanism of virus reproduction has been touched if at all, only with delicacy by most virus workers. Significant contributions have recently been made in this field. Mosley & Wyckoff (67) initially observed the occurrence of rods or filaments in preparations of influenza viruses A and B, an association regarded as significant. Similar structures were seen by Heinmets (41) who considered them a form of the virus, while Chu, Dawson & Elford (19) clearly regarded the long forms as a stage in the multiplication of the virus. In the beautiful electron micrographs of influenza virus in tissue cultures by Murphy, Karzon & Bang (68), every stage from filaments to the individual influenza virus particles was clearly evident. Possibly the spheroid represents the mature "resting" virus, which lengthens in the host cell to a filamentous entity and fragments transversely to form the spheroid again. Somewhat similar forms thought associated with the virus were seen by Dawson & Elford (27) in preparations of fowl plague virus absorbed on red blood cells.

Bergold (14) has observed in preparations of the virus of silkworm jaundice forms grading from spheroids to fully developed rods. Spheroids cloaked in a clearly shown membrane become broad rods eventually escaping the membrane which retains its ghost-like shape. Bergold suggests fragmentation of the insect virus rod, the mature form of the virus in this instance, into subunits that become the spheroid which lengthens again. The micrographs of Black *et al.* (16) of tobacco mosaic virus inside cells throw no light on the mechanism of multiplication of this virus, nor have characteristic intermediate forms in bacteriophage reproduction been clearly demonstrated in the pictures by Wyckoff and his associates (30, 136, 137).

Some of the forms seen in preparations of Newcastle disease and avian leucosis viruses may well represent developmental stages. If the sperm form represents the mature virus particle, the tail might be lost in the process of host cell infection. Elongation of the head piece might then occur, followed

by redistribution and segmentation of the internal material and, subsequently, by transverse division to form short rod-like structures or spheroids.

Density.—Sedimentation of purified viruses in solutions of low molecular weight, i.e., sucrose [Smadel, Pickels & Shedlovsky (103), Lauffer & Stanley (56), Sharp *et al.* (99)], sodium chloride (103), and glycerol (103) has revealed a dependence of sedimentation rate on solute concentration. With vaccinia (103) and influenza viruses (99), the rate varied with time of contact with the solute, giving evidence of a semipermeable membrane susceptible to osmotic effects of solutes of low molecular weight. No such effects were seen by Sharp and associates (96, 99) in studies on the papilloma and influenza viruses in the high molecular weight bovine serum albumin. The densities of the influenza viruses A and B and the swine type in aqueous suspension were estimated (99) as 1.104, 1.104, and 1.100. The value (96) for the papilloma virus was 1.13 and estimates by Elford *et al.* (32) for mumps, fowl plague, and Newcastle disease viruses were 1.10, 1.14, and 1.12, respectively. Bovine serum albumin is not as simply applicable to all viruses as Lauffer (55) found with tobacco mosaic virus. Under proper conditions, however, a linear relation between sedimentation rate and density of the albumin solution gave Schachman & Lauffer (88) the value 1.13. In contrast with the findings with vaccinia and influenza virus, a linear relation was observed likewise with sucrose solutions, yielding the much higher value 1.27. Correlation of the results, however, gave the relatively very high value 1.31 for the wet density of the tobacco mosaic virus rod.

Dry density or its reciprocal, partial specific volume, is generally determined by weighing relatively large amounts of highly purified virus in pycnometers. A procedure requiring but small quantities of virus not necessarily of high purity has been developed recently by Sharp *et al.* (93) by sedimenting influenza virus in heavy water, D_2O [Lépine, Levaditi & Giuntini (57)]. There were no evident changes in the partial specific volume or viral properties of the particles [Kredel *et al.* (53), Svedberg & Eriksson-Quensel (116)] due to heavy water, and the values obtained this way are comparable with those obtained with the pycnometer.

With both dry and wet densities known, water content can be calculated. The values for the influenza viruses A and B and the swine type are 52, 34.5, and 43.3 per cent by volume. The analogous value for the papilloma virus is 58 per cent and, in contrast, that for the tobacco mosaic virus is only 15 per cent.

Another procedure has been developed recently [Sharp (91), Sharp *et al.* (93)] for estimating wet density and water content by measurement of the pellet volume of a known weight of sedimented virus. With this pellet volume and the dry density from pycnometer or D_2O determinations, water content or wet density can be obtained. The resulting values are closely similar to those obtained by the other procedures.

Comment.—The findings in these fields show that viruses contain the materials fundamental to the structure of living organisms. The results of such analyses, while not yet revealing the interrelationships of the various

units, nevertheless have an intimate bearing on the interpretation of the physical evidence of segregation and differentiation of components of functional significance. The physical studies demonstrate that viruses exist as unicellular units; many of the agents within the larger size range are demonstrably organized into an internal structure corresponding to a nuclear apparatus surrounded by a definitely different substance analogous to cellular cytoplasm, characteristics which accord with the chemical findings. Some, if not all, viruses are enclosed within a peripheral membrane-like structure which can be seen in electron micrographs and demonstrated functionally by other physical methods. It is now clear that viruses, at least those causing disease in man, animals, insects, and bacteria, constitute a special group of organisms, and that the unnatural theories to the contrary have been but a fanciful interlude presently of historical import only. This is not meant to imply that the agents causing disease in plants differ from the other viruses; instead, it is evident that solution of the nature of plant viruses has lagged woefully, having progressed but little beyond the realm of conjecture (6) of two decades ago.

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INTERFERENCE BETWEEN ANIMAL VIRUSES[1]

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The observations of many investigators have amply shown that infection of a host (bacterial, plant, or animal) by one virus may, under certain conditions, afford temporary protection against infection by another. (For example, an animal infected with yellow fever virus may be rendered temporarily resistant to infection with Rift Valley fever virus.) The antagonistic action between the two viruses, which results in the dominance of one and the partial or complete suppression of growth of the other, is generally referred to as "interference"; this phenomenon is also sometimes referred to as the "sparing effect," "blocking effect," or "cell blockade."

The clinical observation that an individual attacked by one disease may thereby become refractory to infection with another disease agent goes back many years in the recorded literature. Montaigne, in 1580, stated that "one ill cureth another" and Quier, in 1780, reported that smallpox invariably was a mild disease in children with secondary yaws (1). In 1803, Winterbottom mentions that some African tribes of the Sierra Leone region treated chronic ringworm by inoculating the affected area with herpetic material (1), while Archer, in 1809, reported that vaccination ameliorated the course of whooping cough in children (1).[2]

The earliest clinical observation on the growth of one virus interfering with the development of another is perhaps that of Jenner who, in 1804, reported that vaccinial lesions often failed to develop properly on the skin of individuals with herpetic infections (1).

Such observations, however, were merely of passing interest, and such significance as they may have is derived in retrospect from recent experimental investigations which have led to the discovery and the definite recognition of the interference effect.

Present knowledge of, and interest in, the interference phenomenon stems from observations first made on plant viruses two decades ago [McKinney, 1929 (3); Thung, 1931 (4); Salaman, 1933 (5)]. The first observations on the occurrence of an interference effect between viruses which affect animals are those made independently by Hoskins (6) and Magrassi (7) in 1935. Since that time, numerous observations in this field, which has so much promise of yielding data not only of theoretical interest to biology but also of practical value to applied medicine, have been recorded by many authors.

[1] The survey of literature pertaining to this review was concluded in December, 1950.

[2] It is of interest to note that Dalldorf, Cohen & Coffey (2) have found that under certain experimental conditions, vaccine virus induces resistance in the mouse to infection with *Hemophilus pertussis*.

General Considerations

Animal species and test systems employed.—The interference effect has been observed to occur in a variety of animal species (8). Experimentally, mice, rabbits, guinea pigs, hamsters, hedge-hogs, monkeys, calves, foxes, chickens, and chick embryos have been employed. Tissue cultures have also been used as a tool in the study of this phenomenon [Andrewes (9); Lennette & Koprowski (10)].

General site of the reaction.—The blocking effect has been reported to occur in the central nervous system, the respiratory tract, the viscera, the skin, and the cornea. In the chick embryo, the reported sites of interference have been the chorio-allantoic cavity and the yolk sac (8).

Interference between immunologically-related viruses.—The observations of Hoskins (6) and of Magrassi (7), the first recorded on the occurrence of interference between animal viruses, were made on agents which are immunologically indistinguishable, viz., non-encephalitogenic and encephalitogenic strains of herpes virus in the rabbit (Magrassi) and neurotropic and viscerotropic strains of yellow fever virus in the monkey (Hoskins). Subsequent observations by many workers with a variety of viruses have fully demonstrated that interference between immunologically related, and between immunologically identical, viruses can be induced at will under appropriate conditions.

Interference between immunologically distinct viruses.—In those instances of interference between antigenically related or indistinguishable viruses, the suspicion naturally arises that the effect may have its basis in specific immune processes, especially humoral immunity. However, in most of the experimental work recorded blockade is so rapidly induced, often within a matter of hours, that it is difficult to envision antibodies of any sort as having a role. Also, interference can be demonstrated in chick embryos at a developmental stage in which the function of antibody formation is not yet established [Burnet (11); Burnet, Stone & Edney (12); Enders & Pearson (13)], and antibody formation has not been detected in infected chick embryo tissue cultures [Lennette & Koprowski (10)], even by use of the highly sensitive extra-neural neutralization test in new-born mice (14). Finally, the best evidence that specific immune phenomena are not basically responsible for the interference effect is that blockade can occur between viruses which are antigenically distinct. In this connection, it should be remarked that the greater proportion of observations dealing with interference have been made on systems in which the opposing viruses were immunologically distinct; the recent tabulations of Findlay (1) and Henle (8) illustrate the various opposing combinations in which more than a score of viruses have been studied.

Evaluation of interference.—The facility with which the results of interference experiments can be assessed varies markedly; the methods employed for evaluation must perforce take into account the nature of the viruses under study, and may be simple and direct, or relatively complicated,

depending upon the degree and extent to which differences exist in the properties and characteristics of the opposing viruses.

When both viruses possess about the same degree of pathogenicity for the host, the occurrence of interference may be demonstrated or detected by several procedures. One method, if both agents produce lesions, is to show that the lesions present are predominantly those arising from the action of the blocking virus (15 to 20). A much more common method is to compare the concentration of the presumably blocked virus (or of both viruses) in the tissues of the test animals with that present in the tissues of the control animals. Depending upon the conditions of the experiment, several procedures for quantitating the opposing viruses have been utilized; these are based upon differences in the properties of the viruses, as mentioned above, which permit titration of one agent in the presence of the other. Thus, quantitation on occasion may be simply carried out by using a route of subinoculation which is effective with one of the viruses but not with the other (9, 10, 21, 22). Under other conditions, the use of different animal species susceptible to only one or the other of the opposing viruses may be required [e.g., Ginsberg & Horsfall (23); Lépine *et al.* (24)]. Since susceptibility to infection frequently varies with the age of the animal [cf. Lennette & Koprowski (25)], animals of an age susceptible to only one of the viruses may be used (10). When none of these methods is feasible or desirable, quantitation may be effected by titrating one virus in the presence of immune serum against the other [Lennette & Koprowski (10); Ziegler & Horsfall (26); Florman (27)]. Finally, in the case of those viruses which possess the capacity to agglutinate erythrocytes, assays may be done *in vitro* (10, 26, 28), using the simple hemagglutination technique if only one of the agents possesses hemagglutinating properties, hemagglutination-inhibition with the heterologous immune serum if both possess this property.

Additional procedures to evaluate the extent of interference may be utilized if differences in pathogenicity exist between the two viruses, as when, for example, one has been inactivated by physical or chemical means. The degree of blocking may be measured by death or survival of the test animals [e.g., Hoskins (6); Golub & Wagner (22); Vilches & Hirst (29)], or by an increase in the average survival time [Findlay & MacCallum (30); Lennette & Koprowski (10)]. When the agents produce characteristic lesions, interference can be evaluated by the extent to which such lesions are suppressed (7) or their severity decreased [Andrewes & Elford (31)]; if neither agent gives rise to lesions, sub-inoculation of the tissues of the test animal into suitable animals or into tissue cultures may be necessary.

Reciprocal interference.—In any pair of opposing viruses, the blocking ability is not necessarily an attribute of only one of the agents, since it has been well shown that either member of an opposing combination may interfere with the growth of the other. It does not necessarily follow, however, that interference is always a reciprocal affair; in many instances it has been possible to demonstrate the occurrence of a blocking effect in one direction, but not in the other. In part, inability to demonstrate a reciprocal relation-

ship is undoubtedly due to technical difficulties. The amounts of each virus employed may not be in optimal relationship (10, 26), the time interval between inoculation of the interfering virus and the challenge virus may be at fault [e.g. Vilches & Hirst (29); Rhodes & Chapman (32)], the test species may not be the best suited for the purpose, etc. On the other hand, inability to demonstrate a reciprocal effect may, in some instances, be due to the fact that in certain combinations of opposing viruses, the fundamental mechanism responsible for the interference phenomenon governs the direction of the blocking action, i.e., some unknown biologic difference between the opposing viruses determines that interference will occur in one direction only.

Dual infections.—Inoculation of an animal with two viruses sometimes results not in partial or complete interference, but in a dual infection. Many instances of dual virus infections have been reported (cf. 10, 24, 31, 34 to 37), but Henle (8) points out that, with one possible exception, the viruses parasitized "two different organs or tissues, two different cells within one organ or tissue, or two different structures within one cell"; the exception referred to is the observation of Syverton & Berry (38) that cells of the Shope rabbit papilloma can be superinfected by extraneous viruses. Theoretically, at least, the absence of an interference effect might therefore be attributed to the fact that the agents multiply in different organs or tissues and do not compete in their nutritional requirements; likewise, even though they may invade the same cell, they are able to propagate because each utilizes a different metabolic pathway. Certain observations, however, are difficult to reconcile with such an explanation. Thus, although interference between influenza viruses A and B is readily demonstrable (26, 39, 40), Sugg & Magill (41) report that both viruses can be carried simultaneously through repeated passages in the chick embryo. Similarly, although Henle (8) reports that reciprocal interference occurs between mumps and influenza viruses, Ginsberg & Horsfall (23) observed that these viruses will grow simultaneously in the chick embryo. Gallagher (37) found that equine encephalomyelitis virus and Lansing poliomyelitis virus will propagate simultaneously in the mouse brain, although in the monkey these viruses interfere with each other.

Virus exaltation.—Following inoculation of a host with two viruses, a third situation may obtain, viz., virus exaltation. In this situation, one agent enhances the virulence of the other, so that in its outward manifestations at least, the effect is the opposite of interference.

Vanterpool (42), 25 years ago, reported that inoculation of the tomato with tobacco mosaic virus and potato mottle viruses resulted in a severe infection known as streak. The studies of Gordon and his associates [see literature references in Findlay & Howard (43)] have shown that louping ill virus does not invariably produce encephalomyelitis in sheep, but if both louping ill and tick-borne fever viruses are injected, an encephalitis almost invariably results. Lépine & Marcenac (44) found that inoculation of herpes

simplex virus markedly enhances the virulence of pseudorabies virus for the mouse, and Findlay & Howard (43) have recently reported that both the MEF-1 poliomyelitis virus and the mouse encephalomyelitis virus increase the virulence of the Columbia SK virus for the mouse. Exaltation has been observed so far only with neurotropic viruses, and apparently is due to some pathogenetic property of one virus which facilitates the passage of the other virus across the hemato-encephalic barrier (43).

Some of the Basic Factors Involved

Dosage of virus and temporal relationships.—Interference may occur when (*a*) the blocking virus is injected prior to the one to be excluded, (*b*) when both are given simultaneously, or (*c*) when the interfering virus is administered subsequent to injection of the virus to be excluded. In order to demonstrate a blocking effect under any of these three conditions, the relationship between the amount of virus inoculated and the time element must be observed. Briefly stated, the relationship must always be such that the blocking virus is given some definite advantage over the one to be blocked; failure to favor the interfering virus may result in dual infections (10, 26).

If the blocking virus is administered first, the second virus must be given at some variable period later which is optimal under the experimental conditions employed [e.g., Rhodes & Chapman (45)]. This interval is generally considered to represent the time required for the interfering virus to propagate to the necessary level, since it can be shortened by increasing the concentration of the blocking virus either at the initial injection or by giving repeated injections. The length of the refractory period, during which the blocking potentiality is present, varies from a few days to several weeks [e.g., Schlesinger *et al.* (46); Vilches & Hirst (29); Rhodes & Chapman (32)] in the case of antigenically unrelated viruses; the duration of the refractory state in the case of antigenically-related agents is difficult to delineate because of the complicating factors introduced by the emergence of specific immune phenomena.

The need for allowing the blockading virus some advantage over the virus to be excluded is further shown in those instances in which both agents are inoculated simultaneously. If the same route of inoculation is used, the concentration of the interfering virus must be considerably greater than that of the virus to be excluded if a blocking effect is to be successfully induced. The disparity in the concentrations of the two agents must be greater if the blocking virus is inactive, or propagates slowly, less if the blocking virus propagates at a rate greater than the virus to be excluded. The interfering virus may similarly be given an advantage by inoculating it by a route which allows it to reach and become established in the sites of predilection before the advent of the second virus, which is administered by another route.

This same set of conditions or stipulations applies equally to successful demonstration of interference when the virus to be excluded is inoculated

prior to the blocking virus. In addition, the interval between inoculation of the opposing viruses must be short, so that the virus to be excluded cannot multiply to the level where it can itself give rise to a blocking action.

Interfering effect of inactive virus.—While much of the experimental work on interference has been carried out with the use of fully active (infective) opposing viruses, recent work has shown not only that agents deprived of infectivity by physical or chemical means are still capable of exerting a blocking effect [see (8)], but that the use of such inactivated viruses possesses definite advantages in studying the host-virus relationships.

Perhaps the most obvious advantage is the fact that properly inactivated virus cannot multiply within the host, and hence the exact amount of virus required for a blocking effect can be determined with some accuracy, as has been done, for example, in the recent studies of Isaacs & Edney (47) in which minimal interfering doses of influenza virus were employed. Such studies would not be feasible with active viruses since, although the infectivity of the agent injected can be easily determined, the level which it must attain in the host before a blocking effect comes into play is considerably more difficult or impossible of resolution.

The use of inactivated virus provides a useful tool for investigations into the quantitative relationship between virus and cell which determines interference. At present, however, fully effective use of this tool is hampered by certain gaps in knowledge, such as lack of precise information as to how many viral particles constitute one infective dose, which preclude unequivocal interpretation of data derived from experiments on this aspect of the problem. In this connection, the Henles (48) have found that inoculation of $10^8 ID_{50}$ (50 per cent endpoint infective doses) of influenza virus into the allantoic cavity of the developing chick embryo produces as much virus during the first and only infectious cycle as does inoculation with ten times as much virus, i.e., this is the maximum amount of virus taken up by the allantoic cells. Hence, inasmuch as allantoic fluid virus preparations inactivated by ultraviolet irradiation must contain approximately the equivalent of $10^8 ID_{50}$ to produce practically complete interference, and since the number of cells lining the allantoic cavity has been computed to be between 10^8 and 10^9 [Hoyle (49)], it would appear that each allantoic cell takes up, or accounts for, the equivalent of 1 ID_{50}. Henle (8) believes it reasonable to assume that approximately one active virus particle may initiate infection of a cell, since while studies of influenza virus preparations purified by centrifugation indicate that about ten particles constitute 1 ID_{50} [Friedewald & Pickels (50)], a large proportion of the particles are noninfective because of inactivation during the procedures of preparation and purification, because some are probably impurities derived from the host tissues, etc. He points out, however, that whether all the cells of the allantoic membrane are susceptible is unknown. Isaacs & Edney (47) also point out that any attempt to calculate the average number of virus particles per allantoic cell necessary to produce a blocking effect is confronted with these same unknown quantities, viz., the number of viral particles comprising one infective dose and the number of allantoic cells susceptible to infection.

One objection to the use of active viruses lies in the marked pathologic reactions produced in the tissues of the host, reactions which may impede or even deny access of the opposing virus to the tissue site of the blockading action (51). Inactivated virus is less apt to evoke alterations such as those associated with a living, propagating agent. Nevertheless, it should be kept in mind that subtle effects not discernible by histopathologic techniques may be exerted by an inactive agent. Henle and associates (52) have observed that inoculation of large amounts of inactivated influenza virus into the allantoic cavity of the developing chick embryo hinders further development of the allantois, although no histologic evidence of any change is detectable. In this particular instance, the detrimental effect of the virus is, admittedly, on the susceptible cells which are the site of the interference reaction, but it is theoretically not impossible that in certain hosts and under certain experimental conditions, a similar deleterious effect of variable degree may occur in tissues not so intimately involved in the interference phenomenon, and give rise to the same difficulties engendered by the use of active virus, i.e., tissue reactions which obstruct the passage of the opposing virus from the site of inoculation to the tissue site of the blockading effect.

That the method employed for inactivation may in certain situations be of some import is indicated in recent observations on the viruses of influenza, mumps, and Newcastle disease. These viruses possess a hemagglutinin which is part of the virus particle and which, after adsorption to red cells, is eluted spontaneously (53). This adsorption-elution reaction is considered by Hirst (54) to be essentially enzymatic in nature, and will be discussed further from the standpoint of the mechanism of infection and viral multiplication. In the present connection, the possession, or lack of possession, of enzymatic activity by the virus must be considered in the interpretation of the results of interference experiments [Isaacs & Edney (47, 55); Fazekas de St. Groth & Graham (56)], especially when the results are proferred in explanation of the mechanism of interference. Influenza virus may be used as an example. With the influenza viruses, inactivation has been effected, on the whole, by the use of ultraviolet irradiation. Irradiation must be carefully controlled. If the exposure is too brief, not all of the virus will be inactivated, although enough may be inactivated to interfere, on inoculation into the host, with the infectivity of the active fraction (8). Too intensive exposure may destroy properties of the virus other than infectivity; as irradiation progresses, the following properties are lost in the ensuing order: (*a*) infectivity; (*b*) toxicity; (*c*) blocking capacity; (*d*) adsorption-elution mechanism ("enzymatic action") and virus-bound complement-fixing antigen; and (*e*) the soluble complement-fixing antigen (8, 57). From this sequence it appears that the interfering property can be partially or wholly destroyed without affecting the enzymatic activity, and that loss of this activity entails loss of the interfering activity. The objection to inactivation of viral suspensions by heat or by formalin has been that these lead to destruction of the interfering as well as the infective properties, although "residual interfering effects" (8) have been demonstrated with

influenza (58) as well as other viruses (21, 59) treated by these methods. However, under appropriate conditions and with properly prepared heated material, interference can be demonstrated not only to occur, but to occur in the absence of any enzymatic activity on the part of the virus [Isaacs & Edney (47, 55)]. Heating of undiluted allantoic fluid preparations of influenza virus results in loss of enzymatic activity [Anderson (60); Hirst (61)], although this is not true of all strains [Stone (62)]; however, destruction of the enzymatic activity is assured if the virus is heated (1 hour at 56° C.) in a citrate-borate buffer solution of pH 8.5 (62). Using formolized virus and heated virus inactivated by a slight modification of Stone's technique (62), and determining the enzymatic activity of both types of preparations by Stone's method (62) of titration for inhibitor-destroying capacity, Isaacs & Edney (47) found that heated virus lacking enzymatic activity was a better interfering agent than formolized, enzymatically-active, virus.

Nature of the "factor" or "agent" responsible for interference.—It is generally agreed that the available evidence points strongly to the conclusion that the interfering capacity is a property inherent in the virus particle per se, and not attributable to the so-called soluble antigens of the virus or to substances produced by the action of the virus on the susceptible cell.

The Henles, working with influenza virus, found that the agent responsible for interference is not dialyzable through cellophane membranes (58). Lennette & Koprowski (10), using yellow fever virus in tissue culture, obtained evidence that interference is not attributable to nonspecific antiviral substances arising as a result of multiplication of the blocking virus; heated or filtered preparations of the fluid component or the tissue extract contained no substances inhibitory to the growth of neurotropic agents with which yellow fever virus usually interferes. Additional experiments with the heated or filtered culture materials indicated that humoral immunity is not involved in the interference phenomenon, since none of these materials was found to contain antibodies to the yellow fever virus, although the highly sensitive extraneural neutralization test in baby mice (14) was used.

That the interfering capacity is associated with the viral particle is also indicated by the finding of Ziegler, Lavin & Horsfall (39) that passage of allantoic fluids containing influenza virus through a Seitz filter not only removes the virus but also the factor responsible for interference. Similarly, high speed centrifugation of virus preparations results in removal of both the virus and the interfering property from the supernatant fluid [Henle & Henle (63); Vilches & Hirst (29)], and it has been found that the interfering agent is sedimented at the same rate as the virus (63). The Henles have also observed, with influenza virus, that the adsorption and elution of the interfering agent closely parallels that of the hemagglutinating agent, which has been found to be inseparable from the virus particle [see, for example, Hirst (53)].

Gard & von Magnus (64), studying the "auto-interference" phenomenon, obtained information on the nature of the interfering particle. It has long

been recognized that the infectivity of fluids from embryonated eggs inoculated with concentrated suspensions of influenza virus tends to be lower than that of fluids from eggs inoculated with very low concentrations of the virus. It has been assumed that the low infectivity titers observed are due to the high content in concentrated inocula of inactive virus particles; these inactive particles are present in numbers sufficient to interfere with multiplication of active virus, thus producing the phenomenon of auto-interference. Gard & von Magnus found, however, that auto-interference is due not to the presence of inactive virus accumulated under adverse conditions of storage or handling, but rather to the formation of two different virus particles during the growth phase of the virus in the host (65). One type of particle represents the virus as it is generally known, i.e., it measures about 120 mμ, has a mean sedimentation constant of 660S, has the capacity to produce hemagglutination, and is infective. The other particle is smaller (about 70 mμ), has a sedimentation constant of 380S, also possesses the hemagglutinating capacity but, unlike the larger particle, is noninfective. These findings confirm and extend the earlier the observations of Friedewald & Pickels (50), and fit well with those of Hoyle (49) and of Henle *et al.* (66). Thus, Hoyle found that during the growth of influenza virus, complement-fixing antigens and hemagglutinins, i.e., reactive properties associated with the viral particle, appeared well in advance of infectivity. Similar observations were made by W. Henle and his collaborators, who showed, in addition, that multiplication of influenza virus is a step-wise affair (66), the growth curves obtained resembling the "one-step" growth curves described for the bacteriophages (67). The interfering property appears concomitantly with the hemagglutinating property (68) and thus before infectivity.

Further evidence for the production of more than one type of virus particle comes from Schlesinger's work (69) on the propagation of influenza virus in the mouse brain. Despite the apparent failure of pneumotropic strains of influenza virus to grow in the mouse brain, intracerebral inoculation of active virus produces a "toxic" effect [Henle & Henle (70); Hale & McKee (71)] which has been held responsible for interference with the growth of certain neurotropic viruses (29). However, Schlesinger has found that mouse brain tissue, hitherto considered insusceptible to infection with pneumotropic strains of influenza virus, permits such strains to undergo a single incomplete cycle of growth. Thus, following inoculation, the hemagglutinin and complement-fixing antigen content of the brain increases, but infectivity decreases; the newly formed, immature virus is noninfective.

In summary, the available evidence indicates strongly that the viral particle itself is responsible for the interfering effect. In the case of influenza virus, it has been shown that there exists in the reproductive cycle an intermediate stage characterized by particles which, compared to the fully active, classic virus, are smaller and lack the specific groupings which endow the moiety with the capacity to infect, and hence may be regarded as representing immature virus, precursor to the fully active form. Either of these forms is capable of producing interference.

The Mechanism of Interference

Various hypotheses concerning the basic mechanism mediating interference have been advanced. Some of these explanations appear, in the light of information subsequently gained, to be untenable, while others admittedly do not cover all the observed facts. It is conceivable, however, that different mechanisms may be involved with different agents, and there is as yet no incontrovertible proof that only a single mechanism is involved.

Hypothesis that interference is caused by inflammatory or toxic reactions produced by the blocking virus.—It has been suggested (72) that the ability of lymphocytic choriomeningitis virus to block the growth of poliomyelitis virus may be due to the inflammatory reactions evoked by the former along its routes of travel in neural tissue, thereby impeding the spread of the latter virus to the susceptible cells. That such factors may be invoked cannot be gainsaid, since there is some evidence that the vascular supply, the presence of regenerating cells, etc., are apparently related to resistance against certain virus infections [e.g., Howe & Bodian (73); Taylor & Sprunt (74)]. On the other hand, tissue damage in itself has been shown insufficient to induce interference. Phosphate buffer, protamine, preparations of the somatic antigen of Flexner dysentery bacilli, picrotoxin, and hyaluronidase have been used by Vilches & Hirst (29) in an attempt to simulate the toxic effects and to bring about the blocking effect, produced by intracerebral inoculation of influenza virus (see above); despite the production of brain damage, or the induction of a convulsive state, no interference with the growth of Western equine encephalomyelitis virus could be demonstrated (29). However, the tissue damage produced by these nonviral agents does not in fact simulate the "toxic" effect produced by influenza virus, since in the latter case, "toxicity" is a reflection of a growth phenomenon resulting in the production of an immature, incomplete virus particle (69). Clearly, the virus must exert some subtle effect on the host cell not exerted by the nonviral agents used.

Specific or nonspecific antiviral substances produced by the blocking virus.—The possibility that the blocking effect is due to the action of specific antibodies or of nonspecific substances produced by the action of the interfering virus on susceptible host cells has been discussed above, and it is evident that there is little experimental support for such a thesis.

The penetration hypothesis.—It was suggested by Delbrück (75), on the basis of observations made with bacteriophages, that penetration of a cell by one viral particle results in some cellular alteration which precludes the entry of additional particles. However, later studies (76, 77, 78) have shown that multiple infection of a bacterial cell may occur with closely related strains of bacteriophage, and this hypothesis has been withdrawn [Delbrück (79)].

Hypothesis of blockade of tissue cell receptors.—The basis for this hypothesis was laid in 1941 with the discovery by Hirst (54, 80) and by McClelland & Hare (81) that influenza virus possesses the capacity to agglutinate red blood cells. During the past decade the hemagglutination phenomenon has

been extensively studied, and the present status of our knowledge in this field is well summarized in the excellent reviews of Hirst (53) and of Svedmyr (82); only certain pertinent observations having implications concerning the mechanism of interference are discussed here.

Hirst found (54) that when influenza virus is mixed with red cells, the cells begin to adsorb virus and during the process are agglutinated. Maximum adsorption is reached within a matter of minutes, and subsequently spontaneous release of the virus begins, until eventually practically complete elution of the adsorbed virus occurs. The process of adsorption and elution in some way affects the red cells, since they lose the ability to adsorb any additional virus which may be added, as is indicated by their failure to aggregate a second time. The eluted virus, however, appears to be wholly unaffected—it retains its infectivity, its antigenicity, and its capacity to agglutinate additional red cells. Hirst (54) hypothesized that the adsorption-elution reaction is essentially enzymatic in nature; an enzyme incorporated within the virus particle reacts with the receptor (substrate) on the surface of the red cell; this reaction is a step preliminary to, and independent of, the agglutination of the red cells. With the combination of viral particle enzyme and cell receptor, dissolution of the receptor occurs, and the virus is released to react with additional receptors.

Hemagglutinins have been described for a number of viruses (82, 53), e.g., mumps, Newcastle disease and the influenza viruses. Receptors for those viruses possessing hemagglutinins have been found not only in red cells, but also in a variety of other materials—extracts of red cells, brain, lung, and kidney; allantoic fluid; egg white; serum; human tears; blood group mucins, and ovarian cyst fluid (see 53, 82). Receptor substances similar or analogous to those of red cells are therefore apparently present in a number, and perhaps all, of the tissues and organs of an animal.

The biological implications of such a virus-enzyme receptor system were first suggested by Hirst, who postulated (54, 83) that the first step in infection is the adsorption of the virus to receptors on the surface of the susceptible cell; resultant destruction of the receptor by the viral enzyme constitutes a break in the cellular defense which permits the viral particle to enter the cell and initiate the processes associated with infection. Experimental evidence in support of this concept was based on the finding that influenza virus introduced into the excised, perfused ferret lung was first adsorbed and then eluted in a fashion similar to the interaction between the virus and red cells (83). Similar adsorption and elution of virus was observed in chorioallantoic cells by Stone (84) and Hoyle (49) and in excised mouse lung preparations by Fazekas de St. Groth (85).

Perhaps the most weighty evidence in favor of the virus-receptor mechanism in infection comes from the work of Stone (86). Certain bacterial filtrates, and especially those from *Vibrio cholerae*, possess a potent destructive action on receptor substances (87). On the theory that destruction of the receptors of susceptible cells should increase the resistance of an animal to infection, Stone tested the prophylactic action of *V. cholerae* filtrates

against influenzal infections. Approximately 100 times more virus was required to infect filtrate-treated chick embryos than was required for control, untreated embryos; in the case of mice, approximately 1,000 times more virus was required as compared with untreated mice.

From these considerations of the hemagglutination reaction and the receptor mechanism of infection, it would appear possible that interference might be effected by blockade of the "receptors" of susceptible cells. In other words, interference is due to the inability of the excluded virus to gain a foothold in the tissue cell because the surface receptors of the cell have previously been occupied and destroyed by the interfering virus.

This hypothesis, although attractive, is subject, however, to a number of objections. Isaacs & Edney (55) point out that the adsorptive capacity of the allantoic membrane is considerably greater than the minimal amount of virus required to produce interference; the development of a resistant state, therefore, does not depend upon blockade (or destruction) of all the available receptors. Henle and his associates (52) also found that the amount of virus required to produce interference is considerably less than the total adsorptive capacity of the allantoic membrane for the virus.

The receptor hypothesis has been queried on other grounds also. As has been mentioned above, the Hirst theory postulates the existence of a viral enzyme which, through its action on the cell receptor, destroys it. On this basis, interference would be due to the destruction of receptors by the interfering virus, which would thus leave no receptors for the opposing virus. The results of several recent investigations, however, are at variance with such an explanation as concerns the role of viral enzymes.

Fazekas de St. Groth & Graham (56) found that by the use of "modifying" doses of periodate, cell receptors can be rendered resistant to the action of both the viral enzyme and the receptor-destroying enzyme of *V. cholerae*. Applying this procedure to mice and to chick embryos, they were able to show (56) by four independent methods (including interference techniques) that susceptible cells can be infected through modified receptors on which receptor-destroying enzymes are incapable of action. Conversely, heat-killed virus, which lacks enzymatic activity and therefore is not capable of receptor destruction, is as readily taken up by normal ("unmodified") receptors as is active virus [Fazekas de St. Groth (88); Isaacs & Edney (47, 55)]; that viral preparations devoid of enzymatic activity can produce interference has been brought out elsewhere above. It would thus appear that alteration or destruction of cell receptors is not a necessary concomitant of interference.[3] Nevertheless, a virus capable of inducing interference does,

[3] On the basis of the data summarized in this paragraph, plus several other considerations, Fazekas de St. Groth & Graham (56) suggest that the first step in the process of infection is adsorption of the virus to the surface of the susceptible cell; this union is a "physical combination between two surfaces of complementary pattern, the 'receptor area' of the cell and the viral 'combining area'." Following adsorption, the virus enters the cytoplasm by "viropexis," a passive process analogous to colloidopexis, the uptake by cells of submicroscopical particles. Enzymatic destruction of receptors is not essential.

in fact, produce changes in the susceptible cell. Thus, the Henles (57), using irradiated preparations of influenza virus, all of which possessed enzymatic activity, but in some of which the interfering capacity had been destroyed, found that virus without interfering ability was adsorbed to the cells of the allantoic cavity, but produced no apparent changes in the membrane. Virus with interfering capacity, in contradistinction, not only was adsorbed to the allantoic cells, but also affected their subsequent development, and rendered them resistant to infection with active virus. From such findings, it appears more likely that interference is caused not by alterations in the surface mechanics of the cell, but by intracellular alterations in physiology.

Hypothesis that interference is due to competition for cellular metabolites or enzymes.—This hypothesis [or hypotheses (8)] is based on observations which indicate that interference does not occur during the first stage of infection, i.e., adsorption of the virus to the cell surface, but at some later stage, after the virus has entered the susceptible cell. According to this concept, then, interference is due not to competitive adsorption of virus to cell receptors, but to competition by the opposing viruses for vital cellular constituents.

Reference has already been made to the observations of the Henles (52, 57) and of Isaacs & Edney (55) which suggest that interference lies in some process beyond the stage of adsorption of virus to cell. In addition, several workers have found that interference effects can be diminished or abolished if large challenge doses of virus are given, even at a stage when infection of susceptible cells by the blocking virus is presumably well established (8, 10, 26, 39, 47). This suggests that interference is an intracellular phenomenon arising from competition of the agents for essential cell constituents. Thus, Isaacs & Edney (47), studying the effect of the concentration of challenge virus used on the degree of interference obtained, plotted a curve relating the mean hemagglutinin titer observed to the dose of challenge virus used. It was found that the hemagglutinin titer did not rise uniformly as the concentration of the challenge virus increased, but rose slowly at first, and then steeply as maximum amounts of virus were given. Such results, according to Isaacs & Edney (47), would be expected if interference is due to competition for cellular elements, i.e., when two viruses are competing for the same substance within any one cell, the interfering effect of virus A would be diminished or abolished when the ratio of virus B particles to virus A particles per cell is increased.

What the nature of these essential substances may be is exceedingly difficult to ascertain, since direct studies on the biochemical mechanisms involved in viral propagation face tremendous technical difficulties. By means of indirect approaches, however, some highly important fundamental data have come from the work of Horsfall and his associates.

Horsfall & McCarty (89) found that polysaccharides derived from certain bacteria, especially the Friedländer bacillus type B, exert a powerful modifying effect upon the course of infection in mice inoculated with the virus known as PVM (pneumonia virus of mice). Ginsberg, Goebel & Horsfall (90) have found that the Friedländer polysaccharide also inhibits the

multiplication of mumps virus in the embryonated egg. Only a minute amount of the polysaccharide is required to inhibit the growth of either virus (89, 90).

While such results might be interpreted as due to chemical blockade of receptors on the surface of the susceptible cell, certain other observations militate against such an explanation. First of all, unaltered adsorption of either PVM or mumps virus by tissue cells occurs in the presence of even much higher concentrations of the polysaccharide than those which will effectively inhibit growth of these agents (89, 91). In addition, the action of the polysaccharide appears to be an interruption of the normal growth cycle of the virus—administration of this chemical substance does not reduce the concentration of the virus already present in the cell, but does prevent its further multiplication. Moreover, the multiplication of either PVM or mumps virus can be interrupted as late as four days after inoculation into the host, at a time when all susceptible cells presumably have already been infected (89, 90). These observations, plus the fact that the polysaccharide has no direct action on the virus itself (89 to 92), suggest that the inhibitory effect is mediated by an intracellular process. A further indication that inhibition of viral growth is an intracellular and not an extracellular affair is the observation (90, 91) that the effect of polysaccharides on the hemagglutinating ability of certain viruses cannot be correlated with the inhibitory effect on the multiplication of these same viruses (mumps, PVM, influenza A and B, Newcastle disease). Thus, some polysaccharides which inhibit hemagglutination by one virus have no effect on the growth of that virus, while others which are highly inhibitory to the growth of a virus have no inhibitory effect on its hemagglutinating capacity.

Since the inhibitory effect according to the available evidence is intracellular, then the polysaccharides would appear to control some metabolic pathway required by the virus for its own synthesis. Accordingly, interference would be demonstrable only in those instances in which both opposing viruses must utilize a common metabolic pathway and, moreover, interference should be reciprocal. (While it has not always been possible to demonstrate reciprocity, failure to do so by no means conclusively proves that reciprocity does not occur—there are still many technical difficulties to be overcome, as has been pointed out above. Also, if one of the opposing viruses is facultative in its metabolic requirements, interference need not necessarily be reciprocal.) Similarly, if different metabolic pathways are employed by the opposing agents, no interference should occur, and both agents should propagate essentially unhampered (dual infections).

On this basis, the relationship to the interference phenomenon of the findings of Horsfall and his colleagues is of considerable interest. Thus, the multiplication of both PVM and mumps virus is inhibited by Friedländer polysaccharide, implying that both employ a common metabolic pathway, and therefore the interference phenomenon should occur; Horsfall (92) has recently stated that mumps virus, as would be expected (93), interferes with PVM. On the other hand, the Friedländer polysaccharide does not affect the

growth of influenza or Newcastle disease viruses, and these viruses interfere reciprocally with one another. But, neither PVM nor mumps virus, which are inhibited by the polysaccharide, interferes with the influenza or Newcastle disease viruses, which are not affected by the Friedländer polysaccharide.

It would therefore appear, judging from the results of interference experiments and from susceptibility or lack of susceptibility to growth-inhibiting polysaccharides, that one metabolic pathway is used by PVM and mumps viruses, and another, different pathway by the influenza and Newcastle viruses. A further indication that the metabolic pathway used by these two groups of viruses is different, is the fact that the multiplication of influenza virus is inhibited by another polysaccharide, derived from apple pectin (94).

Thus, in summary, there is evidence that interference is mediated by intracellular processes. Further, in experiments utilizing growth-inhibiting polysaccharides, it would appear that different metabolic pathways are used by different groups of viruses. It is possible to correlate such differences in metabolism with differences observed in interference phenomena, in that those viruses with similar metabolic pathways interfere with one another, whereas those with different metabolic pathways do not. It would appear, in consequence, that interference may be attributable to a competition between two viruses with similar metabolic requirements for a substance essential to the synthesis of both. Whether this substance is an enzyme, a metabolite, or some other cell constituent, is as yet unknown.

Concluding Remarks

What the practical implications of the interference phenomenon might be is in major part still unknown.

On the laboratory side, the fact that interference can still occur 30 days (32) after inoculation of an animal with a virus is of considerable importance. One of the common procedures for determining the antigenic relationship between two viruses is to immunize animals against one virus and then determine, by challenge inoculations, whether resistance has also been produced to the second virus. Obviously, the long persistence of immunologically nonspecific resistance makes it necessary to rule out interfering effects before concluding that any cross-resistance observed is due to similarities in the antigenic constitution of the two viruses (32, 46). Similarly, it is conceivable, although not proved, that difficulties might be encountered in attempts to isolate viruses in laboratory animals infected with latent viruses, or in maintaining stock viruses by passage through such infected animals.

On the side of applied medicine, it is not impossible that interference effects may find application in prophylaxis or therapy. Green (95), for example, has found that ferret-passage distemper virus ("distemperoid" virus) not only produces in the dog a lasting immunity, but also gives an immediate protection based on interference effects. The slow rate of propa-

gation of distemper virus in the host also makes it possible to utilize the distemperoid virus therapeutically by administering large amounts and thereby inducing interference (95).

Recent investigations have shown that certain neurotropic viruses can also interfere with the growth of neoplasms. Levaditi & Haber (96) found that inoculation of avian-pest virus into mice bearing epithelial tumors resulted in necrosis of the growths; bioassays made of such tumors indicated the cells were nonviable. Turner and his associates report (97) that vaccine virus interferes with the growth of mouse sarcoma 180 and retards the development of a transmissible leukemia in mice (98). The Russian spring-summer encephalitis virus has been found by Moore (99, 100) to be capable of destroying mouse sarcoma 180; the oncolytic process was effective, regardless of the age of the tumor or the route of inoculation of the virus. Koprowski & Norton (101), in a comprehensive study on interference between neurotropic viruses and transplantable mouse tumors, present evidence that a number of viruses may adversely affect the growth of neoplasms. It is interesting to speculate on the possible clinical application of such findings.

Finally, investigations into the interference phenomenon should also add to our present meagre knowledge of the processes of viral multiplication. Although such information may be regarded as largely academic, it forms, nevertheless, the foundation for a rational approach to the problems of chemo- and antibiotic therapy.

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INTERFERENCE PHENOMENA BETWEEN PLANT VIRUSES[1]

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Interference phenomena between plant viruses will be considered to include, for purposes of this review, those interactions variously designated as, "immunization," "immune reactions," "protection," "reciprocal protection," "virus antagonism," and "mutual antagonism." With plant viruses, "interference phenomena" may be defined as those phenomena in which a plant or plant part, after being invaded by one virus or virus strain, is rendered immune from or resistant to infection or to invasion by a second or challenging virus or virus strain.

A type of interaction that may be basically different from the usual type of interference, and involving suppression of one virus by another unrelated to it, was described by Bawden & Kassanis (7) who found that severe etch virus prevented the multiplication of potato virus Y and *Hyoscyamus* virus 3 and replaced them even in plants in which they were established. Because of this and certain other known properties of these viruses, it was suggested that this reaction does not indicate relatedness, but rather indicates that the etch virus affects the metabolism of the host cells so that some material, or enzyme system, which is normally present and essential for the multiplication of potato virus Y and *Hyoscyamus* virus 3 is no longer produced.

Still another kind of interaction that appears to differ from the usual type of interference results in what appears to be an increase in the concentration of one virus as a result of the presence of a second. Bennett (12) found that tomato plants produced new leaves that were deformed and necrotic following inoculation with dodder latent mosaic virus. The necrotic stage was followed by recovery with a marked decrease in virus concentration in the recovered tissue. Introduction of tobacco etch virus or tobacco mosaic virus into recovered plants resulted in the reappearance of typical severe symptoms of dodder latent mosaic, in addition to those produced by etch or tobacco mosaic, and the concentration of dodder latent mosaic virus increased markedly and remained at a level much higher than that in comparable plants with dodder mosaic only. A similar relationship between potato viruses X and Y was described by Ross (47) who found that juice of *Nicotiana glutinosa*, Turkish tobacco, or Placid variety of potato, infected with viruses X and Y, may contain much greater amounts of virus X than do juices of comparable plants infected with virus X alone. Differences, which were as much as five-fold, were greater with mild than with severe strains of the X virus.

[1] The survey of literature pertaining to this review was concluded in January, 1951.

Perhaps the most plausible theory that may be advanced to explain this type of interaction would be that it is due to disturbance of the normal metabolic processes of the plant by one virus in such a way as to bring about an increase of materials essential to the reproduction of a second virus. However, it might be that one virus tends to inactivate or destroy substances inhibitory to increase of the second virus.

Certain environmental influences, such as the temperature to which the plants are subjected, are known also to influence virus concentration. Apparently, certain other factors may exert a similar influence. For example, Yarwood (57) reported that bean leaves infected with bean rust and tobacco mosaic may produce concentrations of tobacco mosaic virus estimated as high as 50 times greater than those in comparable leaves with tobacco mosaic alone.

Methods of Testing for Interference

Challenging viruses usually are introduced into diseased plants by one of three methods, (*a*) by wiping leaves with liquids containing virus, (*b*) by graft, or (*c*) by means of an insect vector.

The wiping method provides the most sensitive test for interference. By this method the challenging virus is introduced in small quantities into many locations, usually on mature leaves fully invaded by the first virus. Thus, immediately, small dosages of the challenging virus are in competition with relatively large concentrations of the challenged virus, which would offer the maximum of opportunity for the operation of antagonistic reactions. This method is especially effective with viruses that have the property of producing numerous local lesions on wiped leaves, since in such cases antagonism may be estimated quantitatively on the basis of number of local lesions on healthy and diseased leaves. Unfortunately, only a small proportion of plant viruses are known to produce local lesions on any of their host plants.

Inoculation by graft probably provides a less sensitive test since the challenging virus may have an opportunity to pass into the inoculated plant over an extended period of time, and if union is complete it passes into the phloem through which it is distributed rapidly to various parts of the plant, including the growing points where antagonism may be less pronounced than in mature tissues. Most insect vectors also introduce virus into the phloem and introduction may extend over the period of insect feeding. Thus, inoculation by means of graft or by means of vectors, followed by movement in the phloem, would afford greater opportunity for the challenging virus to come into contact with sites in which it could become established and multiply.

Survey of Recent Studies on Interference Phenomena

Protection of a plant invaded by one virus against invasion by a second virus was reported first by McKinney (34) in experiments with tobacco

mosaic in which plants infected with light-green mosaic showed no change in symptoms after three successive inoculations with yellow mosaic. Soon thereafter, Thung (53) demonstrated that tobacco plants infected with tobacco mosaic virus showed no additional symptoms after inoculation with a variant that caused "white mosaic" when inoculated into healthy plants. Salaman (48) found that tobacco plants fully invaded by mild strains of the potato virus X were protected against further injury by strains that produced marked mottling or other distinct injury when inoculated into healthy plants.

The literature up to 1940 dealing with "immunization" of plants by virus strains has been covered in a comprehensive review by Price (44). In this review, Price stated that protection by one virus strain against related strains was a phenomenon common to all viruses that had been tested. The relatively few instances of failure of one virus strain to protect completely against other related strains were thought to be the result of incomplete invasion of the test plant by the first virus introduced. However, on the basis of results since 1940 in cross-protection tests with a greater number of viruses, it would seem necessary to modify this concept somewhat and to conclude that there is a rather wide range in degree of protection to be found between related virus strains.

Cucumber and tobacco mosaic viruses.—Both cucumber and tobacco mosaic are caused by large numbers of virus strains varying in virulence, host range, and other characteristics. Suitable plants are available for tests of strain interference for both of these viruses by the local lesion technique. In general, strains of each complex have given complete, or a high degree of protection against other strains when tested by the local lesion method. However, McKinney (35, 36) found that tobacco mosaic virus (Nicotiana virus 1) dominated all of its primary and secondary mutants quickly and completely, and that a mild-green mutant sometimes protected only partially against lesion production by the parent virus in Maryland Medium tobacco, although it gave complete protection in *Nicotiana sylvestris.*

More recent results with cucumber mosaic and tobacco mosaic supply further evidence that protection may not be complete in all instances. Fulton (17) identified six isolates, A, B, C, D, E, and F, as strains of cucumber mosaic virus by cross-protection reactions on cowpea and zinnia. In these tests, protection was complete except that some of the cowpea plants with strain B developed small numbers of local lesions following inoculation with strain E and some of the zinnia plants with strain B developed lesions following inoculation with strain C. It was concluded on the basis of these results that under certain conditions complete protection is not obtained between strain B and the other strains of cucumber mosaic virus used in the tests.

In more recent work, Fulton (19) found that leaves of *N. sylvestris* infected with tobacco mosaic virus were not completely immune from invasion by other strains of the virus. Strains that caused necrotic lesions on healthy

leaves also induced local lesions on mosaic leaves. Reinfection occurred, however, only with relatively highly concentrated inocula and only on the dark-green leaf areas, which were shown to have lower virus concentrations than the protected yellow areas. In this case it appears that antagonism of the challenged virus may be partially overcome by increasing the relative concentration of the challenging virus.

Cucumber viruses 3 and 4 and common tobacco mosaic virus were found by Bawden & Pirie (8, 9) and by Knight & Stanley (27) to have a common antigen and similar physical and morphological properties. After McKinney & Fulton (37) determined that certain strains of tobacco mosaic virus cause distinct local lesions on cotyledons and leaves of cucumber, Fulton (18) made cross-protection tests in which he inoculated cotyledons or leaves of cucumber plants with cucumber virus 3 or 4 and later inoculated the same plants with lesion-producing strains of tobacco mosaic virus. The results failed to indicate any protective effect of the cucumber mosaic viruses against tobacco mosaic virus. It was concluded, however, that this did not necessarily indicate complete lack of relationship since a relationship might exist in a degree less than that required to produce a protective or competitive effect.

Tobacco ringspot virus.—Early symptoms of tobacco ringspot on tobacco consist of zonate rings on the inoculated leaves and on a ring of leaves at the top of the plant. These primary symptoms are followed by recovery and the production of leaves that are more or less normal. Price (41) found that virus remained active through three generations of cuttings from recovered plants and that reinoculation with the same virus produced no new symptoms. Inoculation of healthy plants by means of recovered scions gave typical primary symptoms indicating that no protective substances passed from scion to stock. Valleau (55) found that recovered plants, under some conditions, may relapse and again show extensive ring and line patterns. He suggested that this condition might be due to delayed invasion of new shoots.

Price (42) found that ringspot No. 1 and green ringspot gave complete protection against each other, but neither protected completely against yellow ringspot. On the other hand, yellow ringspot protected against ringspot No. 1 and green ringspot. A fourth virus, ringspot No. 2, failed to protect against any of the others and was considered on this basis to be entirely distinct.

In recent tests with tobacco ringspot virus and Pennsylvania ringspot virus, Tall, Price & Wertman (52) found that each virus protected completely against the other, and that these results were in accord with those obtained by serological methods.

Although interference reactions with the ringspot viruses have been nearly complete in degree of protection afforded, Price's (42) results indicate the existence of detectable differences in the potentialities for interference within this group of virus strains.

Potato virus X.—An appreciable range of degrees of interference appears

to exist between the strains of the potato virus X as indicated by the results of several investigators. Smith (51) reported the failure of a mottle strain of this virus to protect tobacco plants against a more severe ringspot type. Bawden & Sheffield (10) found that potato plants infected with virus X were not completely protected against strain B when the inoculations were made by graft, although symptoms of strain B were less severe than when healthy stocks were used. When *Datura stramonium* plants infected with strain X were inoculated with virus B and grafted to the President variety of potato, the second virus produced symptoms. It was found also that plants infected with virus B usually gave one or two necrotic lesions if rubbed over with virus X^s, indicating lack of complete interference by virus B.

Bald (3) found that potato plants infected with a masked strain of potato virus X remained susceptible to infection by more severe strains of virus X, but symptoms of the severe virus were modified by the presence of the masked strain. Also, concentrations of the severe strain and severity of symptoms produced were influenced by the method and place of inoculation. Inoculation of severe strains into immature tip leaves of young potato plants carrying the masked strain caused symptoms almost as readily as on virus free plants, although the symptoms were not always as severe as those on check plants originally free of the masked strain. The severe strain was shown by subinoculation to be present in high concentrations. In contrast, inoculation of the severe strains into lower mature leaves of young potato plants carrying the masked strain resulted in the production of very mild symptoms or none. Subinoculations showed that the severe strains might nevertheless gain access and multiply to some extent, although remaining in low concentration. More or less the same relative conditions with respect to symptoms and virus concentration persisted in the second generation progeny of the two groups of plants, indicating perhaps that equilibrium between two strains may be established at different levels, and once established it tends to remain more or less stable.

Hutton (25) in work with a large number of isolates of the potato virus X found that four masked strains gave complete protection against a severe strain in maturing plants of *Datura stramonium* and *D. metel*, whereas eight masked strains provided no protection in plants of either species, and four masked strains protected almost completely against the severe strain in *D. stramonium* but gave practically no protection in *D. metel*. Degree of protection was thought to be associated with concentration of the masked strains maintained by the plants.

In tests with 10 strains of potato virus X, 4 mild and 6 severe, Mathews (31, 32) found that the protection afforded by mild strains against severe strains on tobacco and *Datura tatula* was complete for all strains tested, excepted for the mild strains T.B.R., X and Y, which gave only partial protection. Both tobacco and *D. tatula* plants infected with either of these strains gave local lesions and systemic infection when inoculated with any of the severe strains but symptoms were less severe than on check plants

with the severe strain alone. The two mild strains differed considerably also from the other strains in serological tests. Each had a strain-specific antigenic fraction and also shared a fraction not possessed by any of the other eight strains.

Ladeburg, Larson & Walker (30) obtained results with several strains of virus X similar to those obtained by Matthews. A mottle type, TBRX, failed to protect completely against any of the ringspot types. Strain B from Duke of York variety of potato gave complete protection against strains X^hR, C-17, and E. Rose, but protection was incomplete against Dak. R., McK. and RW-2-C. Four other mottle cultures protected completely against all of the ringspot cultures. In serological tests, the isolate McK. was found to lack an antigenic fraction possessed by all of the other isolates tested.

Ross (46) states that mild strains of virus X produce no marked symptoms and protect against all tested strains of virus X.

It seems evident from the various results that have been obtained in cross-protection tests with virus X that method and place of introducing the challenging virus markedly affects the results. It seems probable also, from these results, that the strains of the complex are related in different degrees. There seems to be a reasonably close correlation between serological relationships of the strains of virus X and the degree of protection afforded the host plant. It is of interest in this connection, however, that Bawden (6) reported that tobacco veinal necrosis virus was found by serological tests to be related to potato virus Y, but that the two were not mutually antagonistic when present together in the same plant.

Alfalfa mosaic viruses.—Black & Price (14), by the local lesion technique, found that the viruses of potato calico and alfalfa mosaic protected completely against each other on Turkish tobacco and *Nicotiana glutinosa*. Using the same technique, Berkeley (13) inoculated plants previously infected with alfalfa-mosaic virus or potato-calico virus with a virus obtained from pepper and found complete protection against the pepper virus. Oswald (39) found that a recently discovered tuber-necrosis virus and potato calico virus are often found together in the same plant and that juice from such plants transmitted both viruses. It was thought possible also that aphids transmitted both viruses simultaneously under natural conditions. However, presence of the potato calico virus alone in potato plants tended to protect against the tuber-necrosis virus. Protection was not complete since six of 27 calico-infected plants developed local effects on rubbed leaves. None of the calico infected plants developed systemic infection after incubation with tuber-necrosis virus.

Bean and crucifer viruses.—Bean virus 1 and bean virus 2 have almost identical physical properties, but bean virus 1 differs from bean virus 2 in producing less severe symptoms, in infecting a narrower range of host plants and in being seed transmissible. Grogan & Walker (23) found that bean virus 1 completely immunized bean plants against bean virus 2, and bean virus 2 completely or partially immunized against bean virus 1. In further studies

they found that invasion by bean virus 1 completely inhibited the development of a pod-distorting virus in bean plants (24).

Pound & Walker (40) found that cabbage virus B protected apparently completely against infection by cauliflower mosaic virus and that virus A from cabbage protected completely against black ring virus.

Maize streak virus.—The different forms of streak virus in maize, sugar cane, and wild grasses in Africa constitute a group of virus strains or distinct viruses and virus strains in which cross-protection studies should be very interesting from the standpoint of determining the value that such studies have in virus classification. All of the forms are transmitted by the same leafhopper vectors (*Cicadulina mbila* and certain other species of *Cicadulina*), and have not been transmitted by juice inoculation. They produce symptoms that are remarkably similar on the different host plants, but there is a considerable amount of specificity in host range. Storey & McClean (50) showed that a number of forms of streak virus exist in nature. They found that a form from sugar cane, when transferred to maize, offered no protection against the common form on maize. McClean (33) made cross protection tests with the A, B, and Uba forms of the streak virus and found that the Uba form afforded no protection against the A or B form in maize, but that the B form afforded a measure of protection against the A form. In the latter case, plants infected with the B virus 12 days or more before inoculations with the A virus either continued to produce only fine markings, characteristic of the B virus or some of the last formed leaves bore in addition occasional broad streaks characteristic of the A virus. Beyond this the A virus made no progress; the broad streaks, once they had appeared on one leaf, showed no tendency to increase in frequency. Sometimes leaves with broad streaks were followed by leaves with only fine streaks.

Cacao swollen-shoot virus or viruses.—Studies of the swollen-shoot disease in the cacao belt of the Ivory Coast, Gold Coast, and Nigeria in Africa have shown that this disease is caused by a large number of virus strains or distinct viruses and virus strains (1, 2). Tentatively, the forms of virus have been considered strains. They have in common transmission by the mealybug, *Pseudococcus njalensis*, nontransmissibility by juice inoculation, ability to produce disease on cacao, and certain points of resemblance in symptoms produced on cacao.

Crowdy & Posnette (15) in studies of interference between 3 strains of virus designated A, B, and C, found that B afforded some degree of protection against A. The protection appeared to be more effective against infection by insect inoculation than against infection by graft inoculation and more effective in trees in which the internal between respective inoculations was six months than when it was one year. Virus C conferred no protection against A.

Further cross-protection studies with a number of additional strains have given four classes of results (1, 2). These are as follows: (*a*) complete protection by attenuated strain A and strains H and K against strain A; (*b*) incomplete protection in which strain B markedly delayed appearance

of strain-A symptoms; (*c*) no protection conferred against strain A by viruses C, D, E, F, G, J, N, 01, 02, or 03; and (*d*) absence of protection and the formation of a complex in which, when strain A was inoculated into plants already infected with strain M, plants occasionally produced leaf symptoms typical of neither strain. This was assumed to be a visible result of the formation by coincident development of a localized complex which proved to be transmissible intact by the mealybug, *P. njalensis*. More often when plants with strain M were infected with strain A, each virus appeared to follow its respective course of phasic development regardless of the other.

Of the Nigerian selections, 01 and 02 are mutually protective and 03 protects against either 01 or 02. Virus N does not protect against 01 or 02.

Meiffren (38) found two general types of swollen-shoot disease along the Ivory Coast of Africa which he differentiated as Kongodia type and Sankadiokro type. Each gave evidence of being composed of a number of strains. In a test of interrelationships, some avirulent strains were found to confer partial or complete immunity against virulent strains. However, no strain within the Sankadiokro group protected against any strain of the Kongodia group.

Obviously the interrelationships within the swollen-shoot disease complex promises to be varied and complex on the basis of cross-protection tests. Final results of the attempts to classify the different entities on the basis of such tests will be of considerable interest.

Sugar beet curly-top virus.—The strains of sugar beet curly-top virus appear to show a lesser degree of interference than those of any other virus so far studied. Here, also, the host plant appears to be an important influence in determining whether certain strains offer protection against other strains. Wallace (56) found that the degree of protection offered by one strain of virus in an "immunized" tomato plant against infection and injury by a second strain varied widely with the strains involved. For example, plants carrying strain number 3, 8, 53, 55, or 56 showed no increased injury when inoculated with strains 3, 8, or 53. However, symptoms were moderately severe on plants with strain 3 following inoculation with strain 5 or 6, and very severe following inoculation with strain 9. Also, plants infected with strain 56, although showing no additional symptoms following inoculation with strain 3, 8, 53, 55, 56, or 60, produced severe symptoms following inoculation with strain 75. Wallace interpreted these reactions in tomato to indicate that the observed interference was not due to strain antagonism but to immunizing substances that showed a greater or lesser degree of specificity. However, Bawden (5) suggests that they may indicate that curly top is a complex of different viruses as well as virus strains.

More recent results by Giddings (22) indicate an even lesser degree of interference between strains of the curly-top virus in sugar beet. Sugar-beet plants were inoculated with the less virulent strains 2, 4, or 7 and allowed to produce typical symptoms. Approximately three weeks after inoculation, the plants were reinoculated with the more virulent strains 1 or 3. More than 50 per cent of these plants developed severe symptoms within three weeks

after reinoculation; whereas, control plants not inoculated with strains 1 or 3 showed gradual evidence of recovery rather than any increase in severity of symptoms. In tests made to determine whether a more virulent strain protects against a less virulent strain, plants were inoculated with the more virulent strain 3, and a month or two later reinoculated with the less virulent strain 2. These plants showed no evidence of infection by strain 2 but results of transfers from them to plants of the resistant variety S.L. 68, on which symptoms are produced by the less virulent strain 2 and not by the more virulent strain 3, showed that the plants had become infected by strain 2. In further cross-protection tests, in which seven strains were used in 37 of the 42 possible pairings, presence of the second virus was determined in 24 of the combinations. These 24 combinations included all of those for which a satisfactory test is known for determining the presence of the second virus in the infected plant. The best evidence of strain interference was obtained with strains 1 and 3. Plants of the susceptible variety S.L. 842 infected with strain 3 were found refractory to, but not immune from, infection by strain 1. However, plants of resistant varieties infected with strains 3 were relatively susceptible to strain 1, indicating a distinct host influence in the degree of protection offered.

Factors Involved in Interference

Protection by strains of plant viruses against related strains is superficially similar to that between strains of animal viruses, but the two types of phenomena appear to be basically different. Animal viruses incite the production of antibodies in the affected animals that protect against subsequent infection by the same or a related strain, usually for a prolonged period after the inciting agent has presumably disappeared from the animal. So-called sterile immunity to virus infection has not been found in plants and only a few instances of complete loss of virus by plants have been reported. According to Bennett (11), plants of the Columbian variety of raspberry completely recovered with loss of the virus of the alpha strain of leaf curl, but the following season tips from recovered plants were susceptible to reinfection. Also, several investigators have reported that frequently symptomless plants develop from mosaic-infected seed pieces of sugar cane. Forbes & Mills (16) found a high percentage of such plants were free of virus, but that they were susceptible to reinfection, indicating the absence of any residual protective substance in the virus-free plants.

Evidence for the production of protective substances of the nature of antibodies has been presented only in the case of curly top on tobacco and tomato. Tobacco plants regularly recover from the initial severe symptoms of this disease. Wallace (56) found that tomato plants inoculated by means of recovered tobacco scions also produced only mild symptoms in most cases, whereas tomato plants inoculated by means of beet leafhoppers that obtained virus from the recovered tobacco plants were soon killed by the disease. This protection was evident on both tobacco and tomato when inoculated with scions from recovered tobacco or protected tomato. It was sug-

gested on the basis of this evidence that antibodies or antibody-like substances are produced in recovered plants and that these substances are passed from recovered scions to healthy plants through the graft union and protect the inoculated plant from the severe form of the disease. However, both Price (43, 45) and Bawden (5) have expressed disagreement with this interpretation of the data on recovery and protection from curly top. Price (43) first suggested that a mixture of mild and severe strains might be involved, and later, that severity of injury might be correlated with the size of virus dosage that reached the immature tissues before the plant had an opportunity to set up a defense mechanism (45). Bawden suggested that inoculations may have been made with a mixture of an avirulent and a virulent strain, the avirulent strain becoming dominant in the tobacco or tomato plant and the virulent strain being selected in greater quantities by the leafhopper. Thus, transmission by graft from recovered plants would perpetuate the dominance of the avirulent strain but inoculation by means of leafhoppers would give an initial temporary advantage to the virulent strain. Giddings' (20, 21) results indicate, however, that selected strains of curly-top virus are rather stable and no evidence of the presence of avirulent contaminants has been reported.

It is generally agreed, with all viruses so far studied, that plants are protected from infection or invasion only if they are already fully invaded by a virus related to the one being introduced; uninvaded portions of diseased plants remain susceptible to invasion by a second related virus. When two related viruses are introduced into a susceptible plant simultaneously both multiply. Increase may be more or less parallel or one strain may become dominant to a greater or lesser degree. In some such combinations it has been found difficult to separate the component strains. Although it seems evident that protection depends on complete invasion, it does not seem equally certain that complete invasion, or invasion to the natural capacity of the virus, always protects from invasion by a second related virus as indicated by the work of a number of investigators, particularly that of Giddings (22) with curly-top virus.

The theory that has been advanced most often to explain interference between plant viruses is that there is a limited supply of material essential to virus increase in the plant and that when this is exhausted by complete invasion by one virus the plant becomes immune to invasion by all viruses that require the same plant material for increase. Essentially this explanation has been proposed by Kunkel (29), Köhler (28), Bawden (4), Salaman (49), and Valleau (55). Price (44) states that the theory does not explain the "carrier type" of protection in plants which recover from such diseases as tobacco ringspot, in which virus concentrations are lower in recovered than in nonrecovered tissue. However, as Valleau (55) has suggested, differences in virus content may result from differences in amounts of virus-precursor materials in the tissue at the time of invasion. It seems reasonable to expect that leaves that are mature at the time of invasion would have accumulated precursor materials that would be changed immediately to virus, whereas in

leaves immature at the time of invasion virus increase would be dependent more largely on materials formed over a period of time as the leaf developed. Assuming a short lifespan for virus particles, high virus concentrations in tissues invaded when very young might not be reached even though the total quantity of virus produced in them might equal that produced in leaves that were invaded when more mature. Price suggested also that the theory does not provide an adequate explanation of immunity to reinfection in tissues of the same leaf containing different virus concentrations.

Bawden (5) pointed out that there is evidence that some virus strains that reach relatively low concentrations in infected plants protect completely against other strains that reach higher concentrations, indicating that protection may not occur simply because there is a limited amount of virus that a cell can produce. Bawden & Kassanis (7) suggested instead that perhaps unrelated viruses combine with and multiply at different specific sites, whereas related strains combine with the same site. Therefore, if a site in a susceptible cell is already occupied by one strain, then a second strain of that virus would be unable to attach itself and multiply. If there were a limited number of sites, all might be occupied by relatively low concentrations of one strain and thus prevent a more virulent strain from becoming established.

Kavanau (26) proposed that plants are immune to further infection by the same virus or its related strains because viruses exist in infected cells in the form of aggregates which have specific adsorptive properties. Additional virus introduced into the diseased plant does not increase, since it is absorbed immediately by the primary aggregates, thus merely adding to the amount of virus already present in the aggregate. It is proposed that immunity to related strains probably has its basis in the larger number of antigenic groups common to the various strains of a virus. This theory appears to have the advantage of providing for different degrees of interference between related strains since interference would be expected to depend, in part at least, on the number of antigenic groups available for adsorption. It seems possible that viruses like that of curly top, known to be adsorbed readily and rather completely on a variety of proteins in plant juice, may be largely adsorbed also on plant constituents in the infected plant. Free aggregates, therefore, might not be present to adsorb and thus inactivate newly introduced virus of the same or a related strain, and therefore plants would remain susceptible to infection and invasion by related strains.

Summary and Conclusions

The great majority of virus diseases of plants that have been studied extensively are not caused by a single unvarying virus but by a complex of strains that vary in virulence, host range, and other characteristics. There is evidence that interference between the strains varies greatly with different complexes. This variation may extend from a high degree of protection against the majority of the strains of a complex, as with tobacco mosiac virus to little or no protection between any of the strains on certain hosts, as with curly-top virus.

There is evidence indicating, in some cases at least, that the method and site of introduction of the challenging virus, environmental conditions, and species of host plant, may greatly influence the results of cross-protection tests. Inoculation with the challenging virus by wiping the inoculum over the surface of the leaf has given more decisive results than inoculation by graft or with vectors. Apparently, in some cases interference can be partially broken down by increasing the dosage of challenging virus. The challenging virus appears to have a better chance of invading the plant if introduced into very young leaves than if introduced into older mature leaves. This may indicate that the challenged virus does not move into young tissues as fast as growth processes provide tissue capable of supporting virus increase.

How much reliance should be placed in the results of cross-protection tests in attempts to determine relationships within groups of viruses classified as strains on the basis of symptoms, host relationships, vector relationships, physical or other characteristics, remains in doubt. It seems safe to assume, within limits, that viruses that are mutually antagonistic are related; however, it does not seem equally certain that all viruses that do not show mutual antagonism are wholly unrelated. The cross-protection test is of great value in identification of certain virus strains and in grouping related strains within some virus complexes. Probably its value will vary with different complexes. In the present state of our knowledge, it seems probable that it will be of considerable value in classification of viruses if used in conjunction with other methods of determining relationships, but not as a sole criterion of relationships. Certainly its use at the present time in determination of distinct entities in the curly top complex would serve no useful purpose.

Several plausible theories, including competition for materials for virus increase, limited sites for virus increase, adsorption of the challenging virus by virus aggregates already present in the plant, and in one instance production of antibodies or antibody-like substances, have been proposed to explain cross-protection phenomena between plant viruses, but conclusive direct evidence to support any of these theories is lacking.

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POLIOMYELITIS AND POLIOMYELITIS-LIKE VIRUSES OF MAN AND ANIMALS[1,2]

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This review, while centered about poliomyelitis virus, will consider other viruses which, for one reason or another, have been given attention by workers in the field of poliomyelitis. The fact that these agents are considered is not meant to infer that they are related to poliomyelitis virus; in fact recent data tend to show otherwise. A provisional definition of poliomyelitis virus, based chiefly on host range, histopathology, and antigenicity, has been prepared by a Committee of the National Foundation for Infantile Paralysis (1); this should aid in clarifying the issue. The contrary position is maintained by others (2, 3)—that agents antigenically distinct from poliomyelitis virus and with different biological properties may still be taxonomically related to it.

Reviews of work on poliomyelitis virus have recently appeared, including, in addition to comprehensive summaries (4 to 8), discussions of the general problems of running a poliomyelitis virus laboratory (9) and descriptions of chemical and physical methods used in preparing clinical samples for animal inoculation (10). Furthermore, the 2nd volume of the *Poliomyelitis Bibliography* (11) is due to appear in 1951 and will probably cover articles which have appeared from 1945 through 1949, making it unnecessary to duplicate here the complete listings to be found in the *Bibliography*. Consequently, the present review need concern itself only with those results of current research which the author feels he can profitably discuss.

Host-virus relationship in poliomyelitis after natural routes of infection.—In the laboratory this problem can be studied only in animals infected by peripheral routes. The infection need not necessarily be one of the central nervous system (CNS), a point which has been underscored by studies on the growth of poliomyelitis virus in non-nervous tissue (12). When monkeys were fed poliomyelitis virus, serum antibodies appeared early whether or not paralysis developed (13, 14). In fact, when paralysis did occur after feeding, antibodies were usually present on the first day of paralysis, in contradistinction to the delayed antibody development in paralyzed monkeys which had been intracerebrally inoculated. In nonparalytic animals in which antibodies appeared, no lesions could be found in the CNS.

Early antibody development also occurs in man. If one uses the strain producing infection in the patient, antibodies may be demonstrated at the

[1] Aided by a grant from the National Foundation for Infantile Paralysis.

[2] The survey of literature pertaining to this review was completed in February, 1951.

onset of paralysis, and be shown to increase in amount during the next few weeks (15, 16, 17). This is true not only with monkey passage strains, but also with the patient's own acute-phase stool used as a source of virus (18), eliminating the need of "establishing" the strain in monkeys.

The finding of antibodies at the time of onset of the disease means that the occurrence of virus in the blood stream must be reinvestigated, because of the possibility that the essentially negative results (19) have been due to an *in vivo* neutralization of the virus. Only 1 of a series of 111 patients yielded a positive test for virus, although in a study of the experimental disease in one laboratory the virus has been found in the blood of 4 of 10 paralyzed monkeys (20). Studies will have to be made during the period between exposure and onset of disease (at which time antibody production is already under way), or it will be necessary to devise methods for removing antibody from neutralized virus, if such combined virus exists in the blood. The situation may indeed parallel infection of mice with Coxsackie virus (21): a viremia occurs during the incubation period which lessens in intensity as the day of onset of the disease approaches, and coincidentally with the disappearance of detectable virus from the blood antibodies make their appearance.

The chimpanzee has been extensively studied in two laboratories (22, 23, 24) as a model of what may take place in man upon repeated oral exposures to the same and different immunological types of poliomyelitis virus. Following oral exposure or intradermal inoculation of virus, chimpanzees become intestinal carriers of the virus and develop neutralizing antibodies. In some instances, humoral antibodies may be found before detectable amounts of virus appear in the stool. If such animals, previously infected with a certain strain, are again fed the same type of virus, it usually passes through the alimentary tract as though it were an inert substance, and no carrier state results. However, if a different antigenic type of virus is fed, the response is like that of a non-immune host: the animal becomes a carrier of the new type of virus and develops antibodies to it. Interpreted in the light of possible mechanisms of immunity in the human population, the results with chimpanzees suggest that there is strain-specific immunity, and that perhaps repeated asymptomatic infection with different strains of virus is the basis for the increasing immunity which occurs in man with increasing age. It has been repeatedly demonstrated that during epidemic times many healthy intestinal carriers exist, especially in the younger age groups, and it is chiefly on the basis of large numbers of such carriers that one can at present account for the presence of poliomyelitis virus in urban sewage (25 to 29). In man then, a carrier state may follow upon each exposure to a different strain until resistance to a wide variety of strains is finally achieved. However, in view of the occurrence of adult carriers it has been suggested (30) that the duration of immunity to alimentary carriage may not be long-lived, even to homotypic strains, and that repeated carrier states even to homotypic types are necessary for the maintenance of

high antibody levels and immunity to the paralytic form of the disease. In this connection, on the rare occasions when reinfection with homologous or homotypic strains has occurred in chimpanzees (22, 24), the carrier state was relatively short-lived, lasting only for a few days. This suggests that the previous infection, even though not conferring a solid immunity to alimentary carriage, did contribute to early cessation of the reinfection.

One human family has been studied in which multiple cases of infection with poliomyelitis virus occurred twice in a period of two years, each time resulting in a fatality (31). Either the family was infected with two different antigenic types, or the immunity following the first attacks had worn off two years later. The latter possibility appears unlikely if the recent data (32) on the persistence of poliomyelitis antibodies in man has any relationship to immunity. The problem of the distribution of virus in the infected family has continued to receive attention (33, 34, 35). Brown & Ainslie (36) have recently included, in addition to virus tests, antibody determinations both to the Lansing type and to the type responsible for the family outbreak. Their results show that the healthy children in the family respond precisely as the patient (or patients) not only in respect to virus excretion, but also in respect to the development of antibodies to the current family strain. At the time of onset of illness in the patients, the adults already had antibodies both to Lansing and to the family type whereas the children had antibodies to the family type alone. The children, but not the adults, were found to be carriers of the "family virus." It would appear that the adults had had prior infections with both Lansing and family types and were immune to both at the time of the current family outbreak. On the other hand the children, including the paralytic case, had not been previously exposed to the Lansing type, and presumably not to the family type. As would be expected, antibodies to the infecting strain were found early as well as some weeks after virus excretion was detected. If this interpretation is true, one should expect to find in the period immediately following the occurrence of a case that neutralizing antibodies present to the infecting strain will increase in titer within the next few weeks in all members of the family currently infected. Such a rise in titer should occur only in the newly exposed children, but not in the previously infected adults. This would follow from the studies which show that patients already have antibodies to their current infection at the time of onset of disease, and that these increase in titer during the next few weeks (15 to 18).

The fact that antibodies to the family strain appear in most of the siblings at the same time as they appear in the patient emphasizes again, as do the simultaneous virus carrier states, that families seem to be exposed as an entire unit to a common source. This finding, together with the seasonal concentration of healthy carriers (and virus-carrying flies) in the same area as cases, makes one question the commonly held belief that epidemic poliomyelitis may be completely explained on the basis of person to person contact. As has been said before, contact of patients and others with an

"infected place" would yield the same concentration of carriers about a case as could be accounted for by person to person contact. This concept is in agreement with the repeated observations of the simultaneous infection of members of a family with poliomyelitis virus, and supports the view of the widespread seeding of virus in the family through a common vehicle rather than through serial person to person spread within the family (31, 33, 34, 35).

Gear and his colleagues (37) have made the important observation that in an urban native location in South Africa silent infection occurs early in life, as shown by the isolation of poliomyelitis virus from the stools of 4 of 16 infants who were studied during their first year. Apparently the Lansing and non-Lansing types of virus were recovered. The infant from whom the Lansing virus was isolated developed Lansing antibodies as did two others from whom a poliomyelitis virus (untyped) was isolated. More studies to evaluate the significance of the Lansing antibody are needed.

The degree and persistence of immunity in relation to antibodies.—From results with monkeys (38, 39), cotton rats, and mice (31), the degree of immunity, as measured by resistance to intracerebral challenge, is proportional to the level of antibody in the serum. Also in chimpanzees, antibodies and immunity persisted together for at least two years (24).

Several extensive surveys of Lansing neutralizing antibodies have been carried out. Turner and his colleagues (40) believe that the rate at which silent infection with Lansing virus was acquired in Baltimore was constant from year to year during the period 1941 to 1948, being about 20 per cent of susceptibles per year. In keeping with the seasonal incidence of poliomyelitis, the development of Lansing antibodies was limited to the summer months, regardless of the incidence of clinically recognizable poliomyelitis. In tropical and subtropical areas the proportion of positive sera increases rapidly with age, approaching 90 to 100 per cent at 3 to 5 years (28, 41, 42), and sometimes even earlier (43). However, in California cities (41), the percentage of positives failed to increase after the age of 9. The same trends were observed for antistreptolysin titers. There appears to be an inverse correlation between favorable socio-economic status and the early development of antibodies (41, 44). Hammon (30) suggests that immunity in poliomyelitis is temporary and that repeated infections are required to maintain an elevated level of antibodies. However, this may not always be the case and is not in accord with the results obtained in a special survey carried out by Paul & Riordan (32). In this survey, a series of neutralization tests carried out on sera collected in 1949 from a remote Eskimo village in northern Alaska showed that antibodies to the Lansing type, although absent in the age group below 20, were present in high percentage in those aged 20 and above (32), that antibodies to the Brunhilde type first appeared in those over 30, and that antibodies to the Leon strain were present only in those over 40 (43). If current beliefs concerning the specificity of neutralizing antibodies are true, then these data may be interpreted to indicate that

Leon virus was present about 1910, Brunhilde virus about 1920, and Lansing virus in 1930. From the scanty records available it would appear that clinical poliomyelitis was present in this area in 1930, and there is no knowledge of its presence before or since. Results apparently at variance with these were obtained by Clark & Rhodes (45), who studied a small group of Eskimos with poliomyelitis. Of six who had Lansing antibodies in their acute or early convalescent sera, four lost this neutralizing substance completely and one showed a marked drop in level. These results were obtained in neutralization tests using a virus dose of 20 ID_{50}, a smaller dose than has been employed by other workers (32, 40 to 44). In the present state of our knowledge, it is not clear whether the significance of tests with this small amount of virus is necessarily the same as that of tests in which a larger amount of virus has been used. The report of Lansing antibodies in animal sera (46) has also been criticized on the basis of the small dose of virus used in the neutralization tests (42).

Antibody in the nervous system.—Morgan has reported (47) that specific neutralizing antibody (mouse intracerebral test) is present in those areas of the monkey nervous system which bear the brunt of attack by poliomyelitis virus (Lansing strain) and that this antiviral substance may be detected before antibodies are found in the serum. These findings are at variance with those obtained by Sabin & Steigman (48), who used three strains of recent human origin and tested for antiviral substances in the monkey CNS against the homologous strains in monkeys, as well as against the Lansing strain in mice. None was found. As the latter workers point out, it is possible that strain differences account for their failure to repeat Morgan's observations; on the other hand, it is possible that the issue can be settled only by neutralization tests in both monkeys and mice with material derived from monkeys convalescent from paralysis produced by the Lansing strain of virus. Morgan (49) has further reported that vaccinated monkeys, in which antibody is demonstrable in the serum but not in the CNS, may resist intracerebral challenge of a large dose of homologous Lansing virus, and in response to such an injection of virus, antibody appears throughout the nervous system, even in distant areas insusceptible to the virus. To complicate the picture further, monkeys vaccinated with Lansing virus and then paralyzed with a heterotypic virus acquire in the anterior horn high levels of neutralizing antibodies to the Lansing virus with which they were vaccinated. This lends support to the view that antibody may accumulate in the CNS in response to inflammation rather than being manufactured there. Gard's observations of an antiviral substance in nervous tissues of mice following infection with Theiler's encephalomyelitis virus should also be recalled here, even though the substance behaved differently from true antibody in that it could not be freed from the insoluble fraction of the tissue (50).

Immunologic types of poliomyelitis virus.—From the results of cross protection tests and cross neutralization tests, the evidence is clear that at

present three broad types of poliomyelitis virus are known (51, 52). Even though the Brunhilde type is more readily recovered than the Lansing or Leon types, antibodies to all three are widespread in the human population (53). It is noteworthy that within one antigenic type there are differences as to the severity of disease produced by the various strains, even though the virulent and mild strains may have the same virus titer (51, 54). In this regard it has been suggested that strains obtained from mild outbreaks may at times more regularly produce a mild experimental disease in monkeys than strains obtained from patients with severe paralytic disease (55). However, too few strains have been studied for generalizations to be made. Two strains which fall into one antigenic group may differ in other respects also (56). The Y-SK strain, as mentioned earlier, is able to engender antibody production following exposure of cynomolgus monkeys by the oral route. Under the same conditions, the Lansing strain has failed to do so. The formation of neutralizing antibodies in monkeys is enhanced when the antigen in the form of infected spinal cord is incorporated into a water-in-oil emulsion (57).

Complement fixation test.—Many investigators have joined in the search for an *in vitro* test for poliomyelitis antibodies and almost invariably they have been unsuccessful, presumably because of the low concentration of virus in infected tissue. The development of a complement fixation test by Casals & Olitsky (58) is noteworthy, even though it appears at present to be limited to the Lansing type of virus. It had been observed that the Lansing (59), MEFl (58), and Y-SK (31) strains of virus yield lower titers in infant mice than in older animals. For example, the Y-SK strain had a titer of $10^{-1.5}$ in one day old animals and of $10^{-3.6}$ in adult mice, both titrations being carried out simultaneously with the same batch of virus. However, by serial (and sometimes blind) passage of the MEFl strain in three to four day old mice, the titer of virus became even higher than that found in adult mice, reaching the level of $10^{-4.5}$ (58). An antigen was readily prepared from brains of these animals which gave a specific reaction with Lansing type immune serum from mice, cotton rats, and monkeys. As much as the increased titer of virus, the success of this maneuver may rest in the difference in the chemical composition of infant and adult mouse brains in which the virus is usually grown. The development of an antigen to react with the non-Lansing type of poliomyelitis virus remains as unfinished business. Another complement fixation test has been reported (60) which uses as antigen brains of pigs infected with Teschen virus, and as a source of antibodies, cerebrospinal fluid of poliomyelitis patients. Attempts to confirm this report have failed (42). A flocculation test has been reported (61) which seems to depend upon an antigen difficult to prepare.

Host range.—Poliomyelitis virus of all types infects primates, and in addition five strains of the Lansing type, i.e., Lansing (62), MEFl (63), Yale or Y-SK (64), Phillips or Ph (64), and Wallingford or Wfd (65), produce the disease in cotton rats, mice, hamsters and meadow voles. In South

Africa, a new strain of the Lansing type has been recovered which produces paralysis in mystromys, a common veld rodent, as well as in mice and monkeys (37). The Brunhilde type does not infect cotton rats, hamsters, or mice even though it may persist in the brain 5 to 10 days after intracerebral inoculation (66). It is hoped that reports of the adaptation of poliomyelitis virus to the merion (67) and rabbits with a subsequent loss of pathogenicity for monkeys (68) will be confirmed in other laboratories.

The comparative susceptibility of cynomolgus and other monkey species has been studied in three laboratories (69 to 72). By intracerebral inoculation, cynomolgus monkeys are no more susceptible than the commonly used rhesus. However, following inoculation at peripheral sites or simple oral administration, cynomolgus respond more readily to virus. The apparent finding that flies trapped during epidemics produce poliomyelitis preferentially in cynomolgus monkeys is at variance with results obtained with flies experimentally "infected" in the laboratory (43, 73). Desoxypyridoxine has been considered as a factor in increasing the susceptibility of monkeys to infection by the oral route (74). In studies of the host factor in virus multiplication, Lansing (75), Y-SK (64, 71), and Phillips (64) strains were found to have different infective titers in mice, cotton rats, and monkeys. The titer was dependent not only upon the species of animal in which the virus was titrated, but also upon the species used as the source of virus being titrated.

Tissue culture.—Enders, Weller & Robbins have reviewed tissue culture techniques as they pertain to virology (76) and also have reported a fundamental series of investigations on the growth of Lansing and Brunhilde strains of poliomyelitis virus in suspended cell cultures of human non-nervous and nervous tissue (12, 77, 78). In an excellent summation (78) of their work, they state that the growth of the virus in tissue culture causes cell injury and death as revealed:

"(1) by degenerative changes produced in infected tissue fragments in flask cultures and apparent on histologic examination; (2) by failure of such tissue fragments to exhibit normal cell migration when explanted to plasma cultures; (3) by degeneration of newly emigrated cells in roller-tube cultures; (4) by decreased acid production by infected cells. The conclusion that certain of these manifestations of injury are induced as a result of infection by the virus is further supported by the fact that type specific immune serum prevents their development. These phenomena are of interest from two general points of view. First, they leave no doubt that poliomyelitis virus *in vitro* can multiply in cells other than those of the nervous system and cause profound injury of such cells. Secondly, they provide criteria by which the presence of the virus can be recognized *in vitro* and hence afford a basis of technics for isolating virus from patients or animals, for the quantitative assay of virus, for serological typing and possibly for the screening of chemotherapeutic and antibiotic substances. Further study will be required of the reliability and practicability of the application of these phenomena to such ends."

This work has been confirmed and extended (79, 80). The virus has been

grown in cultures of adult human testes (43, 80). Furthermore, in a study of various cynomolgus monkey tissues including brain and spinal cord, growth of the virus was obtained only in cultures of testis and kidney (43). Calf and bull testes failed in this regard. The growth of this supposedly neuronotropic virus in non-nervous tissue correlates perhaps with the finding that the oral administration of virus to monkeys (13, 14) or chimpanzees (22, 23, 24) results in rapid antibody development, by the first day of disease when paralysis occurs, or without any obvious histopathologic involvement of the central nervous system if paralysis does not occur. Comment has already been made of the parallel situation in man in regard to the presence of antibodies by the time the disease becomes clinically overt (15 to 18).

Recent work on properties of the virus.—Little information has been added to that reported in earlier reviews (6, 81). In spite of many attempts, there is as yet no clear evidence that the virus has been obtained in pure form or that it has been visualized in the electron microscope. When such experiments are adequately controlled by subjecting normal tissues to the same procedures of differential sedimentation or fractional precipitation, then similar "purified" material has been obtained (43, 82, 83). Recently anion exchange resins have been brought to bear on the problem with some success (84). Suspensions of such resins with virus absorbed have been used as poliomyelitis virus carriers in an attempt to furnish sufficient bulk for a visual precipitin reaction, but without success (85).

By means of electron microscopy, DeRobertis & Schmitt (86) reported that poliomyelitis virus, or some structure related to it or to the infectious process, could be seen travelling along "neurotubules." These observations must be reinterpreted in view of a later report from the same laboratory (87) questioning the existence of the neurotubules themselves.

Factors influencing the severity of clinical poliomyelitis.—Russell's observations (88, 89) that continuing physical activity after the onset of symptoms predisposes the patient to paralysis has been confirmed and extended (90). This type of information suggests that virus is in the CNS at the time of physical activity, and that the activity upsets the delicate balance between the virus and its host cells. Other possible provocative factors which have been recently incriminated or discussed are (*a*) vaccination with certain bacterial antigens (91 to 95); (*b*) the superimposition of Coxsackie virus infection on poliomyelitis virus infection (96); (*c*) tonsillectomy (72, 97, 98) which may act by exposing a particularly susceptible portal of entry into the nervous system; (*d*) trauma associated with small wounds (99); (*e*) allergic disturbances (100); (*f*) cortisone (101), and (*g*) pregnancy (102, 103, 104). The increased incidence of poliomyelitis during pregnancy may be the result of greater exposure of young mothers to children carrying virus, rather than to the physiological state of pregnancy itself. The extent of discussion would suggest that although the virus of poliomyelitis is a well known entity, the "etiology" of the clinical disease remains an important

field for study. That the overall severity and age distribution of cases in an epidemic is influenced by the immune status of the population is strikingly illustrated by a winter outbreak which occurred among the Eskimos of the northwest coast of Hudson Bay (105, 106). In this virgin population, paralytic cases and deaths were distributed among all age groups (except those under 3) emphasizing that aging alone does not result in a refractory state to the virus. In addition to serum antibodies a factor which is again receiving attention is the virus inhibiting effect of nasopharyngeal secretions (107). Without much experimental evidence, Armstrong (108) has attempted to relate the seasonal behavior of poliomyelitis to alterations in temperature and relative humidity of inspired air. Such alterations, he suggests, may affect the permeability of the portal of entry of virus—presumably exposed mucous surfaces.

Flies.—Work on the role of nonbiting flies as disseminating agents of the virus, particularly in the early phases of summer epidemics, is continuing (109). It is noteworthy that in one epidemic area, two towns which were marked by their poor level of sanitation were found to be populated with virus-contaminated flies even though the people of both towns virtually escaped the epidemic (110). It is often said that there is no direct evidence that virus is transmitted from flies to man. This appears to be a sterile argument, for one can also say that there is no direct evidence that virus is transmitted from one person to another. It seems of importance, at least to this author and to others (8, 111) that the frequently observed contamination of flies with virus [and their proven potential to spread such virus to food (112)] is the only known seasonal factor which can now be correlated with summer epidemics of poliomyelitis. Personal contact is probably responsible for the transmission of the disease during the winter season and during endemic periods when the incidence is low, but this alone is not sufficient to explain the sharp increase in infection during summer epidemics.

The Coxsackie Group of Viruses (C Viruses)

These viruses are included in this review because of the frequency with which they have been isolated in association with poliomyelitis virus during epidemics of poliomyelitis. They bear no antigenic relationship to poliomyelitis virus, nor do they manifest the same tropism for the primate nervous system. Furthermore there is, as yet, no convincing evidence that paralysis in a patient is the result of C virus activity either in the nervous system or in the skeletal muscle. However, as the following discussion will bring out, there are striking similarities in the distribution of poliomyelitis and C viruses in nature and in infected hosts, especially in the alimentary tract of naturally infected patients and of experimentally infected chimpanzees. These similarities have necessitated an intensive study of the C viruses to gain further understanding of this new and widespread group of agents, and particularly to determine their prevalence and action in the presence and absence of poliomyelitis.

Originally isolated by Dalldorf & Sickles (113, 114) from the stools of patients diagnosed as having poliomyelitis, it seems that infection with these agents may provoke symptoms which mimic such diseases as non-paralytic poliomyelitis, aseptic meningitis, epidemic myalgia or pleurodynia (Bornholm disease), influenza, summer grippe, infectious mononucleosis, acute appendicitis, sinusitis, or simply fever of unknown origin. Although only three years have elapsed since the discovery of the agent, a considerable amount of information has been amassed (31, 114 to 131), and only certain aspects will be selected for comment to indicate the trend of research in this field.

The experimental disease.—Infant mice are the most susceptible animals with the degree of susceptibility decreasing from the time of birth (114), particularly to the Conn-5 and Ohio-1 types to which mice are virtually resistant beyond the fifth day of life (118). Although paralysis with florid myositis may be produced with some strains in adult mice, no strain has yet been successfully carried in serial adult to adult transfers. Infection of newborn mice is anatomically manifested by the appearance of widespread acute degenerative lesions of the striated muscle, focal myocardial necrosis, generalized fat necrosis, encephalomyelitis, hepatitis, and pancreatitis (116, 118, 130), with the skeletal muscle being the tissue most constantly affected in our experience. With some antigenic types (Texas-1, Easton-2), the lesions may be virtually confined to the skeletal muscles. With strains of the Conn-5 Type, other tissues may also be involved. The histopathology of the lesions in mice varies with the age of the animals, differences being noted in one-day-old and four- to five-day-old animals of the same colony (21). Thus in one-day-old mice, hepatitis and pancreatitis may be so severe in Conn-5 infection that they result in overt disease and death of the animal before lesions in the muscle and brain develop. When present, the myositis in these younger animals often is localized and mild. In the four- to five-day-old mice, the liver and pancreas are spared and the muscles bear the brunt of the attack.

The quantitative distribution of the Conn-5 strain in different tissues has been determined after inoculation of four- to five-day-old mice (21). High titers were reached by the second day in blood, heart, liver, muscle, intestine, and its contents, and these were maintained through the 8th day, except in the case of blood in which the virus level fell off earlier. Muscle and brain attained the highest titers, and it was in these tissues alone that virus persisted through the ninth day of illness. As with other agents, pathologic changes appeared after virus had reached peak levels. Acute muscle necrosis occurred first on the 4th day, when there was a peak viral concentration in the muscle, and reached maximal intensity on the eighth day. Mice showed signs of disease beginning on the fifth day, with the highest incidence of illness occurring also on the eighth day. After the eighth day the number and intensity of lesions quickly fell off although scattered acute lesions continued to appear for the next four days. With the decreased

myositis there was a concomitant decrease in the incidence of disease. The regenerative phase, which began one day after the first lesions were noted, reached its maximum development on the ninth and tenth days. It is noteworthy that the regenerating sarcoblasts suffered no injury despite high viral concentrations.

Although no clinical disease has been produced in monkeys following the inoculation of C virus, these animals may develop a subclinical infection (118, 132). For example, following the oral administration of the Ohio-1 strain, cynomolgus (but not rhesus or cercopithecus) monkeys became pharyngeal and intestinal virus carriers and produced neutralizing and complement fixing antibodies. The response of the cynomolgus monkey to oral administration of C virus is similar in many ways to that of the chimpanzee (43, 126). In the latter animal, the infection occurs at the subclinical level and is characterized by a brief viremia of 2 to 3 days, a pharyngeal carrier state of 3 to 7 days, and an intestinal carrier state lasting from 12 to 35 days. Antibodies to the strain fed appear in about 12 days and persist for at least one year. During this period it has not been possible to reinfect such animals with the homologous virus. Following homologous challenge by the oral route, virus may be recovered from the stools (passive carriage through the gut) but not at all from the throat. However, with oral exposure to a second (heterologous) type, chimpanzees respond in precisely the same fashion as they did to the first type. The response of these animals is strikingly similar to that which they show following the feeding of poliomyelitis virus (22, 23, 24).

Although one strain of C virus has been grown in chick embryos, most attempts have met with failure. The one success is attributed to the alternating mouse-to-egg passage technique (133). Another strain has been carried in tissue culture (134).

Properties of the virus (43, 118).—Various strains of the agent have been found to pass readily through bacteria-tight filters (EK Seitz and Corning glass fritted UF). No significant loss in titer has been found following such treatment, and this has been true with extracts of stools of patients as well as with suspensions of infected tissues of passage mice. The virus has proved resistant to a variety of agents including ether, penicillin, streptomycin, chloromycetin, and viscosin, and certain of the commonly used bactericidal agents such as 1 per cent Roccal and 5 per cent Lysol have little effect on it. Chlorine in an initial dose of 0.1 per cent or ethanol at 48 per cent also failed to inactivate the virus. The most rapid and satisfactory inactivating agents were 0.1 *N* hydrochloric acid and 0.3 per cent formaldehyde. Suspensions of certain strains of the virus in water were not inactivated at 55°C. for 30 min., but were destroyed at 60° for 30 min. Frozen virus is stable with no significant loss in titer when stored at −20° or −70°C. for long periods. Moreover, infectivity has been preserved when tissues, particularly muscle, have been stored in 50 per cent glycerol or 50 per cent serum at room temperature for at least 70 days, and in an ice box for over one year (135).

Filtration through gradacol membranes of known porosity has indicated the diameters of four different immunological types (Conn-5, Ohio-1, Texas-1 WS-1) to be of the order of 15 to 23 mμ (118, 136). This has been true with virus from mouse brain and muscle as well as from naturally infectious human stool. It is noteworthy that in two laboratories, a strain belonging to a fifth type (TT, Type 1) has passed through filters indicating a particle diameter only half the size of the above (136, 137). Ultracentrifugation in the partition cell of the analytical rotor, or in tubes in an angle rotor, have yielded sedimentation constants of 100 to 160, with approximate particle diameters of 27 to 35 mμ, for the Conn-5, Texas-1, Ohio-1, and Type 1 viruses (43, 118). These figures were arrived at by following the sedimentation of the infective particle or of the complement fixing antigen, both of which may be identical. The discrepancy in the filtration and sedimentation results with the Type 1 virus cannot be resolved at present. This difference in filtration endpoints for the Type 1 and other types may be one more bit of evidence to suggest that these agents which infect infant mice may include a heterogeneous mixture of viral agents.[3] The placing of these agents together in one group should be regarded only as provisional.

Immunological aspects.—Soon after the discovery of the C group of viruses, it was clear that they existed as multiple antigenic types (115, 138). At least 10 antigenic types are now recognized, and only a small portion of the isolated strains have been classified. The four methods in use for differentiating types have been reported recently in some detail (126, 135, 138, 139, 140), the results obtained with any one method being in agreement with the other three. With some modification to fit the virus-host systems involved, the methods are those of classical immunology and include: (*a*) cross neutralization tests, (*b*) cross complement fixation tests, (*c*) cross protection tests in infant mice born of vaccinated mothers, and (*d*) cross protection tests in chimpanzees. In the latter tests using chimpanzees, "infection" hinges upon whether or not pharyngeal and intestinal carrier states can be produced by oral administration of the challenge virus. With regard to cross protection tests in mice, the susceptibility of these animals is short-lived so that in a matter of a few days after birth they become naturally resistant to the disease. Consequently, cross protection tests in which challenge virus is inoculated into vaccinated mice cannot be performed, because the time required for the expected immunity to develop would place the mice out of their susceptible period. But by challenging infant mice within 48 hr. of birth from mothers vaccinated with different

[3] Another suggestion that we are dealing with agents having different biological properties comes from studies on the distribution of virus in animals at the time of acute diseases (43, 118). As mentioned above, the Conn-5 virus consistently produces a mild viremia (titer of blood, 10^{-1}), while the Ohio-1 strain is rarely detected in the blood. On the other hand, with the Texas-1 or High Point strains, there is a marked viremia on the first day of paralysis. In fact, the titer of virus in the blood, about 10^{-7} is second only to that in muscle and exceeds that found in brain, liver, or intestines.

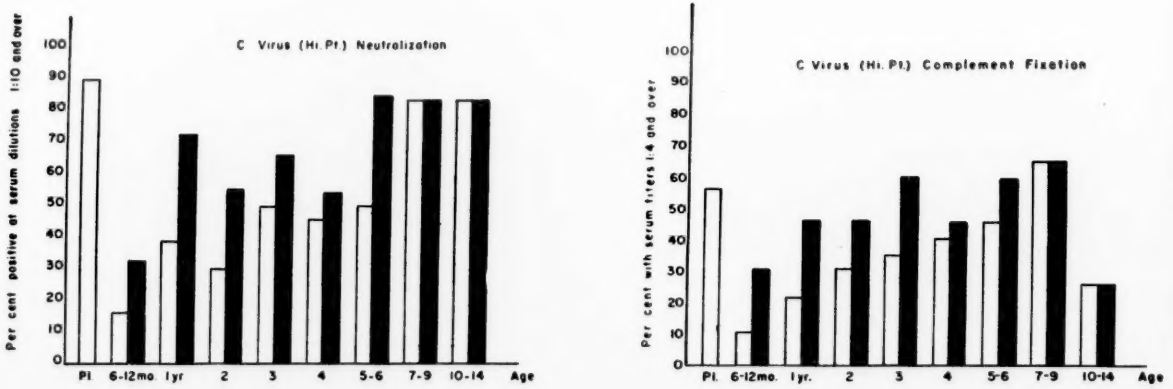

FIG. 1.—Age distribution of neutralizing and complement fixing antibodies in 250 children in Winston-Salem, North Carolina. The columns marked Pl. indicate results on placental sera from 20 individuals. White columns represent sera from children bled in May and black columns sera from the same children bled in November, 1948.

strains of active virus, this procedure may be used as a typing method. In the course of these experiments (140) it was demonstrated that so long as a vaccinated mother lactates, sufficient protection is afforded by a short (one day) nursing period to prevent a non-immune foster litter from being infected by a challenge dose of homologous virus. The immunity conferred by the foster mother is type-specific. Together with the transfer of immunity, there is a simultaneous transfer of complement fixing antibodies from mother to young. It is of interest that in the human population a similar transfer of neutralizing and complement fixing antibodies occurs (see Fig. 1).

The preparation of complement fixing antigens has not yet been standardized. High titer strains present no problem (123, 139, 141, 142); simple extraction and centrifugation yields an antigen of good potency. For low titer strains, we (120, 139) have recommended treatment with protamine sulfate (after Warren) to remove nonspecific interfering substances, and concentration by sedimentation in the ultracentrifuge. Recent work (43, 143) has shown that satisfactory antigens may be prepared even with low titer strains and without concentration if newborn mice are inoculated with large doses of virus and the eviscerated carcasses are harvested 48 hr. later, usually before signs of illness appear. The plate complement fixation test of Fulton & Dumbell (144) has been used for the typing of strains and this work has been reported in detail (139). In addition to the conservation of valuable antigens and sera, one of the great advantages of using the plate method is that the various reactivities of different serum or antigen dilutions with complement alone are taken into account in setting up the test controls. Also the standardization of complement on each testing day becomes unnecessary.

Distribution of virus in nature.—From the data so far collected, there is a marked parallelism between the distribution of C virus and poliomyelitis virus in a community (114 to 118), but perhaps the same could be said of other organisms such as dysentery bacilli if these were studied in similar fashion. Both viruses may be readily isolated from stools of patients and with less regularity from the throat. Family infection occurs with both agents and healthy intestinal carriers have been observed (126, 127, 128). Both viruses have been isolated from urban sewage, yet neither is a constant inhabitant of such sewage (25 to 29, 118). In one year in which tests for both viruses were carried out on serial samples of sewage collected from urban areas in New York, West Virginia, Michigan, Kansas, and Arizona, C virus was virtually absent from three areas but present frequently in two. In one of the latter areas poliomyelitis virus was recovered as well (145). Flies trapped during poliomyelitis epidemics have yielded both viruses (115, 118), but in poliomyelitis endemic times, C virus alone has been recovered from flies (43).

Serological aspects of human infection.—That infection with these agents is commonplace and occurs in the summer season may be seen from Fig.

1 which portrays data from over 200 children in Winston-Salem, North Carolina, who were bled in May, 1948, and again six months later (43, 44). During this period an epidemic of poliomyelitis occurred in the area, having its peak incidence in July. All sera were heated at 56° for 30 min. following which neutralizing and complement fixing antibody determinations were carried out to the local High Point strain of C virus. From the determinations on 20 placental sera, it was found that a high percentage of children start life with antibodies. These are lost during the first year of life and then quickly reappear and rise to the adult level of 80 per cent positive at the age of 7 to 9. Complement fixing antibodies follow the curve for neutralizing antibodies with one sharp distinction, namely, they fall after reaching their maximum at the age of 7 to 9. [Evidence that neutralizing antibodies persist longer than complement fixing antibodies has also been obtained by following individual patients from the time virus was first isolated from them (135).] An appreciable number of healthy children who were devoid of antibodies in the spring developed them by fall. Unlike all other age groups, the 10- to 14-year-old children failed to develop complement fixing antibodies during the summer, lending further support to the fact that they had been previously exposed and rendered immune.

Dual infections in man.—Clinical aspects of infection with C virus have been dealt with by others (116, 117, 119, 121, 122, 125, 127, 128, 146). Of particular significance is the finding that epidemic myalgia (epidemic pleurodynia, Bornholm disease) may be due to strains of one immunological type, the Conn-5 (121, 125, 131, 146). Infection of laboratory workers has been reported (121, 147). Simultaneous occurrence of both poliomyelitis and C virus in the same patient has been found (18, 116, 120, 127). In one explosive outbreak, a group of patients who entered the hospital consecutively during the peak of the epidemic was selected for detailed study (96). Of 36 patients (28 paralytic and 8 nonparalytic) studied for the presence of both poliomyelitis and C viruses in their stools, 20 were found to be excreting both viruses, 7 poliomyelitis virus alone, 6 C virus alone, and 3 were negative for both viruses. That these patients were actually infected with C virus and not merely carrying the agent in the intestinal tract is evidenced by the serological data. Of 26 patients excreting C virus, 23 responded with a rise in neutralizing antibodies to the Easton-2 strain or to the strain isolated from the patient. Of 15 patients from whom C virus could not be isolated, only 3 responded with antibody rises. It was found that with the rise of antibodies to C virus during the month after onset, there was a concomitant disappearance of C virus in the stools. In two paralytic patients studied, antibodies to both poliomyelitis and C viruses increased during the four weeks following the onset of illness (18).

C viruses and poliomyelitis viruses are antigenically distinct and manifest neither serological cross reactions nor interference with each other, as far as reported (118), even with strains isolated from the same paralytic patient (43). Serum of proven potency from animals hyperimmunized

against the patient's strain of C virus failed to neutralize in monkeys the strain of poliomyelitis virus simultaneously isolated from the patient. The converse was also true, namely, hyperimmune antipoliomyelitis serum produced against the patient's own strain did not neutralize the patient's C virus strain.

Spontaneous Encephalomyelitis Viruses of Mice (Theiler's Viruses)

Although these viruses are not included among the poliomyelitis viruses (1), there are some striking resemblances between the infection originally described by Theiler and human poliomyelitis (148). The original type of virus has been recovered in laboratory stocks of mice throughout the world, and is designated the TO virus; it occurs in the intestinal tract and feces, and upon intracerebral inoculation produces paralysis in mice without signs of encephalitis. The second type, of which the FA and GD7 are standard strains, may be less common, having been isolated in stock mice only in two laboratories. These isolations have usually been from mice inoculated intracerebrally in the course of other work (149, 150), and there is only a single instance on record in which it has been recovered from an uninoculated and spontaneously ill animal. Studies on the distribution of the FA strain in mice have not produced any evidence for multiplication outside of the nervous system. The disease which this virus produces is characterized by an encephalitis, although myelitis may also occur, particularly following intraperitoneal inoculation. Work on the purification of this strain has continued in Gard's laboratory, and recent electron micrographs indicate that the virus is a spherical particle with a diameter of 28 mμ (151). Hemagglutination has been reported with the GD7 strain (152, 153, 154).

Because of the latency of these agents in stock mice, they may be accidentially picked up in the course of work with other neurotropic viruses, or in attempts to pass viruses to mice. When such contamination of the murine-adapted Lansing strain of poliomyelitis virus occurred (150), the contaminated Lansing virus was freed of FA virus by treatment with FA hyperimmune serum, or by passage through the monkey, a host which is not susceptible to the FA virus. FA virus was readily obtained from the mixture of strains by serial passage of brains of mice showing signs of encephalitis only, or by passage through chick embryos.

In view of the frequency with which virus is harbored in the intestines and the low incidence of paralysis, TO infection has been suggested as a natural model for poliomyelitis. One of the obstacles to pursuit of this lead has been removed by the development of stocks of mice free from TO virus by foster nursing of newborn mice on albino rats (which do not harbor the virus) (155). Feeding 0.5 ml. of a 10^{-5} concentration of TO-infected brains (this dose contains about 10 ID_{50} by the IC route) was sufficient to produce an intestinal carrier state in the TO-free mice. Such mice also showed a greater susceptibility to TO virus (156). For example, titrations by the

intraperitoneal route in young TO-free mice yielded ID_{50} values of $10^{-4.3}$; in mice of the same age from the normal colony, the titer was less than $10^{-1.0}$. Titrations by the IC route within the first four weeks of life gave the same values in mice from either colony; with increasing age, however, the titers were lower in the normal mice than in the TO-free mice. A series of fundamental observations on the spread and multiplication of virus in young (one week) TO-free mice and in the normal stock has been made (157): (*a*) After IC inoculation, virus multiplication was restricted to the CNS in both stocks. (*b*) After oral administration, virus grew first in the intestinal tract, later in the brain, and lastly in the spinal cord in both strains. No virus was recovered from the muscles or lymph nodes. (*c*) After subcutaneous inoculation, virus did not grow in normal stock mice, but in TO-free mice it multiplied in the muscles close to the site of inoculation and later in more distant muscles and in the CNS. Virus did not multiply in the intestinal tract. (*d*) After intraperitoneal inoculation also virus did not grow in normal mice, but in TO-free mice it spread throughout the animal, although it did not multiply in skeletal muscle. Thus the localization and multiplication of TO virus in the tissues of the mouse vary with the route of inoculation, and are also dependent upon the immune status of the stock. The pattern after oral administration (primary involvement of the alimentary tract and CNS) is strikingly similar to that observed for poliomyelitis virus in fatal human cases. Multiplication of TO virus in murine muscle was found after subcutaneous inoculation but not after the other routes employed, including a natural route of inoculation, the oral one. On subcutaneous inoculation, paralysis occurred first in the extremity in which virus had been inoculated. The invasion of the spinal cord along the peripheral nerves and the localization of virus primarily in the motor neurons supplying the inoculated extremity may not be the explanation of these results, for virus multiplication did not regularly take place first in the spinal cord. Von Magnus raises the question of the local multiplication of virus in the muscle as being a "provoking factor" to account for the frequency of paralysis in the inoculated limb.

With dependence upon IC inoculation of TO-contaminated stock mice for conducting neutralization tests it has not been easy to demonstrate neutralizing antibodies to TO virus, although a prolongation of the incubation period by several days has been noted. However, satisfactory neutralization tests may be performed by intraperitoneal inoculation of one-week-old mice from TO-free stock (157). While none of the sera from the TO-free colony contained neutralizing antibodies, these were quickly produced (by the tenth day) when animals were fed small amounts of virus (about 100 IC ID_{50} doses). The antibody content of serum of ordinary stock mice is at the adult level at birth (1:32), drops to 1:2 at three weeks of age and then reaches adult level again at three months. Mice become spontaneous carriers at three weeks when antibodies are at their lowest level. As already mentioned, paralysis is a rare occurrence in such mice. These findings bear

a marked resemblance to the situation in human poliomyelitis, where the antibodies drop to a low level within a year after birth and then rise at rates which vary in different parts of the world depending presumably upon the degree of exposure to virus. Exposure at a time when maternal antibodies have fallen to a point low enough for infection to take hold may result in an inapparent infection, perhaps because the maternal antibodies, although low, are nevertheless present in adequate concentration to limit the infection to the intestine. This mechanism suggests itself as a possible means of preventing the virus from invading the CNS.

Encephalomyocarditis Virus (EMC, Mengo Encephalomyelitis, Columbia-SK, MM)

This family of agents—all immunologically related strains of the same virus—is given ancillary consideration in this chapter because it has been confused by some with poliomyelitis virus. The classification of the various strains in relation to each other (158, 159), and as separate entities from the poliomyelitis viruses, has recently been elucidated (31, 158, 159). The name encephalomyocarditis or EMC virus has been suggested as being descriptive of the pathological lesions in experimental animals, and at the same time it clearly separates this virus from association with poliomyelitis virus (158). Work on EMC virus has shown that because a disease in mice may at times be manifested by paralysis indistinguishable from that produced by murine-adapted strains of poliomyelitis virus, the etiological agents are not necessarily biologically related. Thus in monkeys the EMC group produces, in addition to neuronophagia similar to that seen in poliomyelitis, a diffuse polioencephalomyelitis involving the entire CNS, such as is never found in poliomyelitis. Also, in rodents the widespread focal necrosis of the cerebral and cerebellar cortex, as well as the extensive necrosis of the entire spinal cord, are different from that of poliomyelitis (158). Jungeblut, who first discovered this virus in 1940, has recently summarized his views (3). He still holds to the position that Columbia-SK is a mutant of Yale-SK virus, a point of view which is at variance with that of the author and others (1, 31, 158, 159). A cooperative study between Dr. Jungeblut and the author was undertaken to determine whether Columbia SK had arisen from Yale SK strain, and the data (31) proved to the author's satisfaction that the two agents are antigenically distinct, a conclusion which has been confirmed in other laboratories (160, 161). The recovery of EMC virus from a chimpanzee which died in Florida in 1944, and the observations of antibodies in rats trapped in the area several years later, has led to the suspicion that the origin of the Columbia SK virus may have been in the Florida cotton rats into which the Y-SK virus had been passed. The latest member of the EMC group was added in 1947, when the Mengo strain was recovered in Africa from man, monkey, mongoose, and mosquitoes (162, 163). Although this virus may infect man (164), present evidence suggests that it does so only rarely. On the other hand it may be more widely distributed in rodents

(158). Work on the EMC family has been aided by the observation of Hallauer (165) that sheep erythrocytes may be agglutinated by the virus, a finding which has been confirmed and extended in several laboratories (166 to 169).

Teschen Virus (Encephalomyelitis Virus of Swine)

This virus has been placed in the poliomyelitis "family" by several workers, and for this reason alone is being reviewed here. Attempts to justify such a relationship have not been successful, and it is now held that the virus should not be so classified. Work with Teschen virus has been recently reviewed (7, 170). The disease was first reported in Czechoslovakia in 1929, and has been reported in other parts of Europe, including Eastern Germany, Austria, Hungary, Switzerland, and recently a strain has been isolated from an outbreak on the Island of Madagascar (171). The disease has never been known to occur in North America.

Recent work has attempted to classify Teschen virus in relation to other members of the neurotropic group (172, 173). Its size by filtration through gradacol membranes was found to be between 10 and 15 mμ. Pathologically the disease in pigs is a diffuse encephalomyelitis with severe involvement of the cerebellum, thalamus, basal nuclei, hippocampal gyrus, Ammon's horn, cerebral cortex, and anterior horns of the spinal cord. This widespread distribution in the brain is in marked contrast to the highly selective distribution of lesions in the brain in poliomyelitis. In Teschen disease, the lesions are primarily neuronal, and the inflammatory exudate is striking in the almost complete absence of polymorphonuclear leucocytes. Both in character and distribution the lesions resemble those of Japanese B encephalitis in man, and louping ill in the pig and monkey more than those of poliomyelitis (172).[4] The host range is sharply limited, and no susceptible host other than the pig has been found, although mice, guinea pigs, rabbits, several species of monkeys, hamsters, cotton rats, meadow voles, eggs, tissue culture, and newborn mice and hamsters have been tried (171, 173). Monkeys and rodents which had been previously inoculated with Teschen virus were as susceptible as controls to Lansing strains of poliomyelitis virus (173, 175). No interference could be demonstrated in mice between Teschen and Y-SK poliomyelitis virus, FA, or EEE viruses. Mice immunized against Teschen virus were as susceptible as controls to Y-SK and Lansing poliomyelitis virus, Theiler's TO, GD7 and FA strains of mouse encephalomyelitis, and St. Louis, EEE, and Japanese B encephalitis viruses. Teschen hyperimmune serum does not react with Teschen virus antigen in a complement fixation test (the usual titer of the virus in pigs is only 10^{-4}), and no relationship

[4] Verlinde (174), in studying the pathology of the lesions of canine myelitis which is found in dogs in the Netherlands, concluded that these resembled poliomyelitis lesions more than do those of Teschen disease. Just as there is no evidence for any etiological relationship between poliomyelitis and Teschen disease, so there is none for poliomyelitis and canine myelitis.

between Teschen and EEE, WEE, SLE, Japanese B, and West Nile viruses could be shown using Teschen hyperimmune serum and the above virus antigens in complement fixation tests (173). Attempts to confirm the report (60) of a complement fixation test for poliomyelitis using Teschen infected CNS as antigen have also failed (43, 173).

Viral and Rickettsial Registry

The American Type Culture Collection has set up a Viral and Rickettsial Registry[5] from which there may be obtained representative strains of all the viruses referred to in this review with the exception of Teschen virus which may not be brought into the United States. Other agents are, of course, also available.

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MICROBIOLOGICAL DETERIORATION OF MANUFACTURED MATERIALS[1]

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Introduction

As a result of attack by microorganisms, many types of manufactured products evidence deterioration in transport, storage, or use. The severe service conditions often encountered by military equipment, and the high standards of performance and durability demanded of such equipment, have intensified interest in the over-all problem of deterioration within recent years. Much work has been carried out in respect to the problems of corrosion, the effects of light, the influence of temperature extremes, and numerous other factors. No phase of the subject, however, has received more serious attention than has that of deterioration by microorganisms. Textiles, leather, plastics, paper, adhesives, glass, and many other types of items and materials of construction have lately been investigated with respect to microbial degradation, and numerous basic studies have been carried out relative to one or another aspect of the behavior of microorganisms.

Since 1945, one of the principal, active organizations for collection, integration, and dissemination of technical information on all forms of materiel deterioration has been the Prevention of Deterioration Center of the National Research Council. Within its files exist thousands of reports on the special topic of microbiological degradation. Because of space limitations, the present review can necessarily refer only to key papers, to subjects not adequately treated elsewhere, and to the most significant recent developments. Persons interested in information more detailed than here presented on any particular topic are invited to consult the Center's *Prevention of Deterioration Abstracts* and, in the case of specific problems, to contact the Center for direct technical assistance.

Examination of numerous individual cases reveals that, in the origin, development, and solution of a problem of deterioration of a manufactured item by microorganisms, certain general types of questions are soon or late recognized as important. Work on a microbiological deterioration problem is usually initiated in response to a report from one or more individuals to the effect that some item has been observed to exhibit an impairment of some useful property in service and that microorganisms are either visibly present or are thought for one reason or another to be responsible for the

[1] The survey of literature pertaining to this review was concluded in January, 1951.

[2] The Prevention of Deterioration Center operates under contract N7-ONR-291, Task Order 27, which is supported jointly by the Navy, Army, and Air Force.

difficulty. Depending on the nature of the item in question, any one or more of dozens of different properties may be influenced, e.g., strength, color, water repellency, flexibility, viscosity, electrical resistance, adhesiveness, optical efficiency, legibility, odor, etc. In some cases the complaint will be that, whether or not microorganisms are actually causing trouble, users suspect microbial growth and hence distrust the serviceability of an item or even suggest discarding it. In a few cases, personnel fear a health hazard as a result of visible fungus growth on items of equipment.

The following types of questions are liable to arise: (*a*) Is the problem genuinely serious in the sense that it occurs commonly, occasions large financial losses, involves risk of physical injury to individuals, or is of unusual military significance? (*b*) What are the criteria and the methods of measurement by which one may recognize the problem and measure its severity as it occurs in practical situations? (*c*) Is the difficulty really due to the activity of microorganisms? (*d*) If microorganisms are active, are they causing degradation of only one or a few of the components present in the item or formulation? If so, which are the susceptible and which the resistant components? (*e*) Can many different kinds of microorganisms bring about degradation under experimental conditions, or are there only a few? Of those known to be active under experimental conditions, which ones, if any, are actually present and clearly causing damage to the item under practical exposure conditions? Do these forms have any particular or unusual requirements or preferences in respect to conditions for growth? Are they geographically localized? (*f*) What is the chemical mechanism by which degradation occurs? (*g*) What devices, materials, or procedures of a preventive or remedial nature can be used, and how can their effectiveness be tested?

The present review deals only with the questions in categories (*a*) through (*f*). The last is the principal topic of numerous specialized and detailed reports and is therefore excluded here. In some problems it is not possible to arrive at absolute answers on the basis of presently available data. A clear recognition of the areas of doubt and ignorance is, nevertheless, equally important as a guide for future experimental work.

Textiles and Cordage

Although the importance of the damage and losses of textiles and cordage during World War II has often been emphasized, in the United States the damage caused by microbiological attack on fabrics, ropes, and other cellulosic materials is still reliably estimated to be several million dollars annually.

Various aspects of the subject of microbiological deterioration of textiles have been reviewed by Thaysen & Bunker (88), Fargher (12), Marsh (56), and others. Until about 1940, intensive investigation of textile deterioration was limited to only a few aspects of the general problem. Much of the information in the rather voluminous older literature is fragmentary and inconclusive.

In his review of 1947, Marsh (56) attempted to summarize not only the then more recent discoveries but also such important and conclusive facts as could be gleaned from the earlier literature. Because of the availability of that review, and in order to provide within the space limitations of the present paper an adequate discussion of the significant papers published since 1947, attention is here directed more particularly to recent rather than to older papers and to the manner in which earlier conclusions have been altered or extended within the past four years.

Cotton fiber has been shown experimentally to be readily decomposed by many fungi. In 1949, Marsh and co-workers (57, 58) materially increased the available information in this field by a report describing tests on numerous fungus isolates and summarizing in some detail the earlier information on the subject. Evidence in regard to the tendering activity of bacteria on cotton fibers, on the other hand, is still rather limited.

Among the fungi, the ability to cause strength loss of cotton has been found to occur in all or almost all of the numerous isolates from such common mold genera as *Alternaria*, *Chaetomium*, *Curvularia*, *Fusarium*, *Gliomastix*, *Helminthosporium*, *Humicola*, *Memnoniella*, *Stachybotrys*, and *Trichoderma*. Among the group of other mold genera in which a lesser number of isolates have been tested, cellulolytic activity has also proven to be very common, although as a result of many tests it is believed to be entirely absent from fungi of the order Mucorales and from many of the penicillia. Active isolates have been obtained from 10 out of the 14 subgroups of the genus *Aspergillus*. Information on the lower Phycomycetes in this regard is almost completely lacking. It is generally assumed that many of the wood-inhabiting Basidiomycetes must be capable of degrading cotton cellulose, but very few tests seem to have been made with them.

Information is still somewhat meager and conflicting on the subject of just which of the fungi, in particular types of practical exposure situations, are actually present in growing condition and causing damage on deteriorating cellulosic textiles. One school of thought has relied heavily on the use of isolation techniques (100), while another believes that such methods can yield only highly questionable results and that direct microscopic observations of the characteristic structures of the various organisms *in situ* are essential (57, 105). The former school appears to find an extremely wide variety of common mold types to be of frequent occurrence on cotton fabric in above-ground exposures (69). *Memnoniella* is mentioned as being of particularly frequent occurrence in tropical locations (100). The latter school lays particular stress on the importance of sunlight as an inhibitory factor in fungus growth on above-ground fabrics. *Leptosphaeria*, *Diplodia*, *Diplodiella*, and other angio-carpous forms are believed by them to be of common occurrence in prolonged sunny exposures, whereas *Alternaria* and *Cladosporium* are said to be common in the early stages of such exposures (57).

In humid storage or other humid, shaded exposures, *Aspergillus*, *Penicillium*, *Memnoniella*, and numerous other fungi not common in situations involving direct exposure to sunlight are to be found. Most of the fungi

thought to possess cellulolytic activity are common in soils, and although direct observations on the point are limited, it is generally assumed that most of these and many others are active in rotting cotton and other cellulosic textile fibers in soils. Levinson (50) has reported that the bacteria capable of decomposing cellulose make up a heterogeneous group including members of the genera *Cytophaga*, *Vibrio*, and *Cellulomonas*. The spore-forming aerobic bacilli do not seem capable of attacking cellulose.

In general, there is little evidence that the fiber-deteriorating fungi are geographically localized. White *et al.* (69, 100) present such evidence as is available. Whereas *Memnoniella echinata* is characteristically tropical in distribution, the fungus *Stachybotrys atra* (to which it is known to be so closely related in structure and physiology as to raise the question of whether the two species are actually distinct) occurs commonly in temperate climates. *Cladosporium*, which has been shown by various workers to be favored by lower-than-average temperatures (58), was isolated frequently by Pady *et al.* (67) from the air at 3,000 to 5,000 ft. over arctic and subarctic Canada, whereas spores of *Aspergillus*, *Penicillium*, and Phycomycetes were conspicuously absent.

Recently, information of a physiological nature has been accumulating on two of the genera of interest to fiber-deterioration workers, *i.e.*, *Chaetomium* and *Myrothecium*. Buston & Basu (15) have recorded certain interesting observations concerning the influence of the composition of the culture medium on perithecial formation by *Chaetomium globosum*. On media containing sugars as a carbon source, a low level of soluble sugar in the medium was essential for the production of perithecia. Jute extract and certain other natural products stimulated both growth and sporulation of the fungus on a sugar-containing medium. A similar response could not be obtained by the use of any of nine of the well-known members of the B vitamin complex, nor could it be duplicated by growing the fungus in the presence of other fungi. It would appear that *C. globosum* requires for perithecial formation some unknown "fruiting substance" which under some, but not under all, conditions reproduces itself in sufficient or optimal quantities.

Mandels & Norton (53) have studied the influence of various factors on germination of the spores of *Myrothecium verrucaria*. In view of the unusually rapid and vigorous growth of the fungus on a simple filter paper-mineral salts-liquid medium as described by Greathouse *et al.* (34), and considering the successful cultivation of the fungus over a period of several years in many different laboratories, the requirements for a high level of spore germination turned out to be rather surprisingly complex. In spite of numerous trials with combinations of sugars, amino acids, salts, and vitamins, no purely synthetic medium capable of producing a high percentage of spore germination was found. Media made with certain types of agar or with glucose plus yeast extract permitted very high germination.

Darby & Goddard (20) found *M. verrucaria* to be strongly aerobic, with no trace of fermentation detectable in pure nitrogen. The respiratory quotient was close to unity even at an oxygen concentration of 1 per cent.

The enzymatic complex, "cellulase," has been the subject of numerous papers over a period of years. But it has been difficult to work with, and only within very recent times could any very satisfactory progress be reported toward the understanding of it. Greathouse (33), in the early period of World War II, obtained an active cell-free preparation of the enzyme. Reese, Siu & Levinson (70) have shown that several noncellulolytic as well as cellulolytic fungi produce an enzyme or enzyme complex, called by them "Cx," which is capable of hydrolyzing carboxymethyl cellulose and certain other cellulose derivatives of low degrees of substitution at the 1,4-β glucosidic linkage. The activity of the enzyme is independent of the degree of polymerization, a result in good agreement with Greathouse's report (33) that action of *M. verrucaria* on hydrocelluloses is independent of the degree of polymerization over a large range. The pH-activity curve for Cx exhibits maxima which differ according to the organism used as a source of the enzyme preparation.

Siu *et al.* (79) tested the susceptibility to microbiological attack of several substituted cellulose derivatives. They have reported that, as long as there is at least one firmly bound substituent in each anhydroglucose unit, the resulting derivative is not susceptible to attack. Greathouse (33) has obtained earlier results generally in good agreement with this conclusion. In tests of substituted sugars, Siu and co-workers (79) and Greathouse (33) found cellobiose octa-acetate and penta-acetyl glucose to be resistant to fungus attack, whereas Abrams (1) reported *C. globosum* to grow readily upon both of these materials and to digest them.

Greathouse (33) and Abrams (1) both report that cotton subjected to attack by fungi displays increased solubility in sodium hydroxide, microscopically visible notches being dissolved out of the attacked fiber by alkali. The longer the period of incubation, the lower is the concentration of alkali required to bring about the effect. The appearance of flax fibers tendered by microorganisms is shown in photomicrographs in a recent paper by Clegg (18). The characteristic fuzzy and frayed appearance seems to be generally similar to that pictured by Abrams (1) for cotton attacked by fungi.

White *et al.* (100) have described the microscopically visible features of growth of the fungus *M. echinata* in a cotton fiber. The fungus grew rapidly in the lumen of the fiber and produced a corroding or pitting action in the secondary fiber wall. *Humicola* sp. and *M. verrucaria* were said to bring about the same type of attack. Siu (80) later described much the same sort of attack for *M. echinata*. He generalized to the effect that fungi produce this type of histological picture, while bacteria cause an etching and corroding from the outer surface inward.

While there is earlier evidence to suggest that the phenomena as described by Siu (80) do actually occur in some such fashion as he described (23, 84), it seems more than probable that the clear-cut differences in mode of attack by fungi and bacteria will ultimately be found to be related to the species of microorganisms and the conditions under which damage occurs. The fungus pitting illustrated by Abrams (1) for *C. globosum* occurred from the outside of the fiber inward, and Marsh & Bollenbacher (57) present photo-

graphs of *Cladosporium herbarum* growing along the outside of the fiber rather than in the lumen.

For use in microbiological deterioration studies, Mandels & Siu (54) have suggested the measurement of oxygen consumption in a differential manometer of the Barcroft-Haldane type. The apparatus described by these authors is of a macro-scale type and is claimed to be simpler in manipulation than certain other respirometers.

Dean *et al.* (22) compared the changes in various properties of unbleached 8-ounce cotton duck on weather-exposure racks at New Orleans, Louisiana, with the changes observed in samples of the same fabric scoured and bleached prior to exposure. Exposures were begun at two-month intervals throughout a year, and each exposure was continued for a year. The unbleached fabric lost strength more rapidly than the bleached, particularly when the first few weeks of exposure were conducive to mildew growth. The authors explain their results in terms of Fargher's (12) "mildew capacity" concept, which involves the ideas that different fabrics in the gray state contain different amounts of nitrogen, salts, and other essentials required for the growth of microorganisms; that they support fungus growth well or poorly according to the amounts of these materials present; and that scouring, bleaching, and leaching by rain during weather exposure may reduce the amounts of these materials present and thus decrease the "mildew capacity" of the fabric.

Cellulosic plant fibers other than cotton are in almost all cases known to be susceptible to strength loss under the influence of microorganisms. Manila hemp, flax, jute, and ramie have all been shown experimentally to be readily decomposed by one or more fungi. The first two are known to be disintegrated in their individual component cells by the noncellulose-decomposing fungus, *Aspergillus niger*. Coir fiber, with its unusually high lignin content, is reputed to be highly resistant, but ramie, contrary to several undocumented public statements, seems from what evidence is available to be susceptible (14).

With respect to the types of microorganisms capable of causing deterioration, wool has not been as extensively investigated as cotton. White *et al.* (98) have provided recent experimental evidence and a review of pertinent information. These authors have concluded that woolen fabrics as placed on the market—perhaps with the fiber slightly but not seriously modified by prior processing—are less subject to fungus attack in storage and use than are cottons. To date, the only fungi reported as clearly proven capable of causing loss of strength in wool, in the absence of autoclaving or other sensitizing treatment prior to inoculation, appear to be a small number of dermatophytes or ringworm fungi (77, 98). Autoclaving renders the fiber subject to tendering action by numerous fungi otherwise incapable of attacking it. It has been commonly believed for many years that wool is more readily attacked by bacteria than by fungi. The fact that wool rots readily in soil contact is considered by some to indicate the action of bacteria.

Mandels and co-workers (55) have presented evidence on the microscopic structural changes in wool degraded by *Microsporeum gypseum* and have

summarized previous literature relating to the effects reported by various authors with other organisms. Numerous reports in the literature indicate that microbiological damage to wool is accompanied by dissolution of the scale cells of the fiber. Mandels and co-workers (55) find, on the contrary, that *M. gypseum* and the bacterium, *Pseudomonas* sp., bring about major strength losses with little or no evident damage to the scale cells. The degrading action takes place on the cortical cells and on the cementing substance between them. Shema & Appling (78), in a later paper, illustrate the disappearance of scale cells in microbiologically damaged wool fiber.

Goldsmith (29) found that a cell-free proteolytic enzyme from *Actinomyces* sp. rapidly digests protein fibers made from peanut protein and casein fibers but that it has only a very slow action on wool and on soybean-protein fiber. Ter Horst (87) claims "Vicara," a protein fiber derived from zein, to have inherent mildew resistance. Stahl and co-workers (83) studied the digestion of fibrillar proteins by *M. gypseum* and found the following decreasing order of susceptibility: collagen, feather, hoof, wool, horn, horsehair, mohair, and silk.

To sum up the present status of knowledge of the microbiological deterioration of textiles, it is clear that a number of fundamental advances have been made in the past ten years. It is equally clear, however, that great gaps exist in our fundamental knowledge of the problem and that most of the recently acquired fundamental information has as yet exerted exceedingly little influence in the realm of practical control measures. The present situation appears to require, therefore, a redoubling of effort toward both the acquisition of new fundamental information and the prompt application of new fundamental facts to the solution of practical problems.

Paper

The microbiological degradation of paper may be divided into three phases: that occurring in the raw material, chiefly wood; that occurring in the partially processed pulp; and that occurring in the finished sheet paper. There seems little doubt according to Lewis (51) that the loss of raw materials, peeled or unpeeled logs, due to microbiological attack during seasoning and storage, constitutes a very important economic problem. Microbiological problems, such as slime formation during the handling of wet pulp in the mill, likewise require expensive and bothersome preservative steps. Stitt (86) in 1948 estimated that loss due to slime in mills producing groundwood-sulfite specialties was at least $1.00 and probably more than $1.50 per ton of production. The loss from slime in the newsprint mill was estimated to be at least $2.67 per ton and probably of the order of $6.00 per ton.

Attack by microorganisms on finished sheet paper, if difficult to estimate and probably not of extreme economic importance, nonetheless is bothersome and in isolated cases may assume proportions out of keeping with the actual dollar value of the paper itself. Consider among many possibilities the complete uselessness of maps, printed on paper of low water repellency

and insufficient fungal resistance, when exposed to tropical conditions, or the frustration and possible wartime dangers attendant upon packaging papers which do not resist microbiological attack and moisture and which therefore spill out their valuable contents when the package is degraded.

Probably little specific work has been done to isolate and identify the organisms causing degradation in piled pulp logs. Such organisms probably include most of those found damaging to living trees and to newly felled trees in the forest. Nason, Shumard & Fleming (65) in 1940, Sanborn & Gillotte (76) in 1941, and Smith (82) in 1941 summarized information on the organisms encountered in paper mills. Those most frequently found include the coliform types belonging to the genera *Escherichia* and *Aerobacter;* the nonsporebearing bacterial species belonging to the genera *Micrococcus* and *Pseudomonas*, and to the poorly defined genera *Achromobacter*, *Flavobacterium*, *Alcaligenes*, and *Cellulomonas*; the sporebearing bacterial species such as *Bacillus subtilis*, *B. mesentericus*, *B. megatherium*, *B. cereus*, and *B. mycoides*, and various thermophilic and anaerobic types; filamentous organisms belonging to the genus *Actinomyces* and special groups such as the iron bacteria; pseudo-yeasts and yeast-like fungi such as *Torula*, *Oidium*, and *Monilia*; and filamentous fungi of the mold type including *Penicillium*, *Aspergillus*, *Citromyces*, *Fusarium*, *Cephalosporium*, *Botrytis*, *Trichoderma*, *Alternaria*, *Cladosporium*, and *Spicaria*. Many other fungus types, such as *Acrostalagmus*, *Chaetomium*, *Trichothecium*, *Thamnidium*, *Gliocladium*, *Mucor*, *Torulopsis*, *Clonostachys*, *Sporotrichum*, and *Stachybotrys*, occur more or less frequently. Some of these species produce slimes; others destroy cellulose fibers, mildew stocks, discolor pulp and paper products, rot felts, aggravate corrosion difficulties, or form objectionable odors in pulp systems.

The composition of slimes varies from mill to mill and from place to place in a given mill, but usually bacteria are the most important single group of slime formers. Fungi may be important in splatter slime but are not usually found in submerged slime, since they do not grow well when completely submerged. Yeasts are found occasionally but seem unimportant, and while algae are sometimes encountered and may under certain conditions be important, they usually cause little trouble since they do not thrive in the absence of light. Protozoa are frequently found, particularly in strawboard white waters, but their presence is fortuitous and they play little or no part in slime formation. Probably the most important slime-forming bacteria are the capsulated bacilli.

Although all pulp-stock systems furnish at least some nutrients, groundwood systems are especially rich in this regard, with strawboard systems also furnishing good support. Sulfite or rag systems are relatively poor in nutrients but will support some bacterial growth. The nutrients available in pulp stocks consist chiefly of mineral salts, sugars, extractives, starches, and cellulose.

Finished paper presents somewhat different problems in microbiological attack, depending upon the use to which it is put. The damage to papers in libraries is one of defacement rather than destruction. Book paper in

libraries has been investigated by several workers including Beckwith, Swanson & Iiams (7). These investigators isolated from old books and manuscripts 55 fungus cultures, including the genera *Alternaria*, *Monilia*, *Aspergillus*, *Penicillium*, *Mucor*, *Stemphylium*, *Hormodendrum*, *Fusarium*, *Chaetomium*, and *Byssochlamys*. *Penicillium* and *Aspergillus* were encountered most frequently.

With regard to such paper items as ammunition containers, corrugated paper and packing pieces, labels, caseliners, battery paper, mine detector cases, instruction manuals, message pads, log books, and like materials used chiefly during wartime, there seems to be little doubt that microbiological attack occurs and assumes importance in proportion to the severity of exposure. The organisms, chiefly fungal, appear to be quite similar to those found on textile materials under similar conditions. It must be remembered, however, that the effect of moisture alone on common varieties of paper probably causes much loss of strength before the effect due to organisms becomes appreciable (81).

Leather

The susceptibility of leather to mildew, aside from plaguing technologists for a long time, has demonstrated, especially during World War II, the damage that microbiological attack on this commodity can inflict upon the Military. In addition to depreciating appearance and causing great economic loss to industry and the public, this phenomenon proved a very real problem to the Military in terms of men and effort required to maintain serviceability of leather-containing items both while in storage and in actual use. Such infections if occurring in enclosed areas, as in a leather case containing an instrument, serve as the focus of further deterioration. The products of microbial metabolism, particularly the water resulting from respiration, create a more humid condition favoring the corrosion of metallic components, the fogging of photographic and optical lenses, and the spreading of further contamination to other materials receptive to fungal infection.

The factors generally considered operative in leather deterioration may be grouped into three categories: (*a*) the reactions with water, termed hydrolytic; (*b*) the slowly changing acid-alkali balance and altered buffering capacity effects, sometimes termed collectively as aging; and (*c*) microbiological attack. The tanning treatment, essentially one of immobilizing reactive chemical points in the native hide, is utilized to impart greater inherent resistance to leather so as to minimize these deteriorative forces. This processing is commonly carried out either with vegetable tannins or with inorganic mineral tannage chemicals, one of which utilizes basic chromic sulfate.

Vegetable-tanned leather is generally assumed to be more susceptible to mycological attack than is chrome-tanned leather, partly because vegetable-tanned leather is less resistant to water even after treatment with the customary greases and oils and partly because the substituents used in vegetable tanning contain more readily available and more suitable nutrients

for fungi. Because of the greater inherent resistance of chrome-tanned leather to fungal attack, and water, it is thus less vulnerable to the hydrolytic and microbiological types of deterioration. The results of Mandels & Siu (54), applying respirometric techniques in an effort to assess the relative microbiological susceptibility of various materials, elucidate further the fact that chrome-tanned leather exerts a greater fungistatic influence against *A. niger* than does vegetable-tanned leather.

Because of the naturally occurring acidic condition of leather, usually pH 3 to 4, bacteria are seldom found operative unless some physical damage causes a pH shift to conditions more optimal for bacterial metabolism and growth. The molds, and occasionally yeasts, attack leather and comprise the major group of agents operative in microbial deterioration. Most of the fungi found to attack leather belong to the very common genera, *Aspergillus* and *Penicillium*. Other organisms encountered in the various phases of leather technology have been compiled by Colin-Russ (19) and Musgrave (64).

The deterioration of leather by microorganisms is somewhat different from that indicated for other fabric materials. There is seemingly no direct deteriorative effect but rather the indication that fungi play a more secondary role in leather degradation. The damage brought about in the physical and chemical properties of vegetable-tanned leather as a result of fungus attack was investigated by Kanagy, Charles, Abrams & Tener (44). Although there is no appreciable deterioration of the hide substance, these workers found that the fats, waxes, greases, and organic oils, incorporated in the leather by treatments following tannage, are metabolized in preference to the proteins of the hide. As a consequence of this removal of conditioning agents, there is an increase in stiffness, a loss in tensile strength, and a decrease in stretch at the breaking point, together with a weakening of grain surface and an increase of cracking tendency.

Kanagy, Seebold, Charles & Cassel (45), in their further investigations on leather exposed to the optimum conditions for mildew growth, differentiate between the deterioration due to hydrolytic action and that attributed to microbiological attack. They postulate, as the principal cause for loss in tensile strength, high temperature and high relative humidity, the combined effects of which result in hydrolytic action. The extent of such action is significantly dependent upon the pH of the leather. Removal of grease and oil from leather, accomplished by the assumed secondary action of mildew, serves to allow more intimate contact with water, intensifying hydrolytic action. As pointed out by these workers, mineral oils may be preferable for maintaining flexibility of leathers designed for tropical use, since mildew does not remove oils of this type.

Confirmation of the secondary nature of the role exercised by mold in leather deterioration has been further established by Barghoorn (4) as a result of his histological studies of mildewed leather specimens. It is concluded that the development and survival of mold on leather is at the expense of substances incorporated during tanning, or subsequent conditioning,

and that such attack is not accompanied by destruction or even invasion of the collagen matrix of the hide substance. The deterioration study by Sachs (74) on mildewed Marine Corps shoes further confirms these conclusions and mechanisms of action.

A review of information pertaining to fungus-proofing measures for leather indicates an emphasized need for the development of leather-making materials invulnerable to mildewing organisms and of extending this prerequisite characteristic as well to the thread, adhesives, and other materials associated with leather and leather products.

Paints

The ability of fungi to attack various paint films and to survive on such protective coatings has long been a recognized problem among paint technologists. Because of this susceptibility, the beautification and protective purposes of paint films are soon defeated, especially in regions where optimum conditions of warmth and moisture are provided for fungal attack. Unless protective films are inherently fungicidal, or are rendered sufficiently poisonous, molds will imbed in such surfaces and break down the organic compounds in an attempt to satisfy their food requirements. This occupation of mildewing organisms, aside from producing spotting and undesirable discolorations, thus results in a disrupted continuity of paint surfaces. While of particular importance to the Military, since it pertains to the development of camouflage paints and their maintenance, the deleterious effect, from the point of view of sanitation, of this type of contamination is also a matter of concern for dairies, laundries, abattoirs, hospitals, breweries, bakeries, canneries, distilleries, etc.

The organisms responsible for paint deterioration include various species of *Aspergillus*, *Penicillium*, *Cladosporium*, *Pullularia*, and *Phoma*. From the extensive examination of many mildewed paint samples, Goll & Coffey (30) isolated 30 organisms belonging to these genera plus some species of *Alternaria*, *Cylindrocephalum*, *Cephalosporium*, *Stemphylium*, *Trichothecium*, *Spicaria*, and *Saccharomyces*. Since *Pullularia* was isolated in greater numbers than any other by Goll & Coffey (30) and by Vicklund & Manowitz (95), this organism is considered the major deteriorative agent and the most common cause of paint mildew. Additional evidence presented by Reynolds (71) shows that *Pullularia* is responsible for serious deterioration of painted surfaces in southern Florida, quite aside from and previous to general climatic injury.

Salvin (75) has shown that linseed, soybean, and fish oil vehicles, including a linseed-tung-soybean vehicle, permit the growth of paint-mildewing organisms, demonstrating that the nature of infection and degree of mildew damage is primarily dependent upon the pigment portion of the paint composition. The inhibition of fungal growth was found by Salvin to be a direct function of the surface area or fineness of zinc oxide. He noted that a fine-particle zinc oxide was particularly effective in inhibiting growth. But even though zinc oxide was found to be inhibitory when in contact with

the fungus, its action is only fungistatic since the organism, when transferred to a more suitable environment, was capable of growth. Because of the impending scarcity of zinc pigments, as pointed out by Vannoy (94), it becomes increasingly important that other pigments possessing inherent anti-mildewing properties be made available for use in paints.

The methodology and interpretation used by paint technologists in a study of the phenomenon of mildew and the determination of mildew resistance must be treated with some caution. The problem is complicated by such factors as weathering, temperature, light, leaching and chalking effects, and their influence on painted surfaces, together with their individual and combined relationship to the mildewing problem.

Plastics and Plasticizers

Rubber and plastics are among those materials generally classified as having a reasonably high resistance to attack by microorganisms. They may not, however, be termed completely inert, since under certain combinations of circumstances at least some members of the class are damaged. Microbiological resistance assumes great importance in the field of electrical and electronic equipment, where plastics and rubber are used widely and where the presence or absence of fungi and moisture, either within the plastic structure or bridging between structures, may decide whether or not the equipment will operate under tropical or subtropical conditions.

Recent work at Rensselaer Polytechnic Institute by Luce & Mathes (52) indicates subvisual growth of fungi to have an effect directly on the electrical properties of plastic surfaces. But this view is not without objectors, and it is not yet clarified whether damage to electrical properties may be attributed primarily to fungi or to moisture (5, 28, 48, 49, 52, 89). There appears no doubt that moisture alone, with certain types of materials, is able to account for breakdown of electrical properties (49). There also appears little or no doubt that fungi alone, under certain conditions, are capable of accounting for measurable electrical breakdown of some plastic materials (52).

It is necessary, in the case of plastics, to differentiate between attack on the polymeric material itself and attack on the materials used as fillers, plasticizers, coatings, etc., as well as to recognize whether the microorganisms are merely growing on the surface debris or are obtaining nutrition from the plastic itself. Often the use of a fungus-susceptible filler, such as wood flour or chopped fabric, leads to the growth of fungi or bacteria when the filler has absorbed sufficient moisture. The polymer itself may be completely unaffected. Mica, asbestos, and glass fillers appear not to contribute to such susceptibility.

While a considerable body of literature exists on the susceptibility to microorganisms of plastics compounded with fillers, plasticizers, and other modifiers, the attempt is made here to use chiefly data applicable to the pure plastics only. These data are summarized in Table 1 and are based on such tests as pure culture agar tests, soil burial, humidity cabinet tests, and tests of electrical properties.

The *Modern Plastics Encyclopedia* for 1949 (61) lists the fungal resistance of 54 plastic adhesives and of 46 plastic coatings. No direct references are provided for the sources of the information, and since there are some conflicts with information from sources cited here, it is suggested that the *Encyclopedia* be consulted for these data. The same publication might well be consulted for detailed information on the structures, compositions, and trade names of the many plastics and plastic products.

The literature on the fungal susceptibility of plasticizers consists predominantly of reports by Molnar & Leonard (63), by the National Bureau of Standards (92), and by the Bakelite Corporation (contract with the Boyce-Thompson Institute) (96). The information in these reports was summarized by Brown (13). Approximately 150 compounds or preparations were tested in the original work in which fungal resistance was measured against pure cultures.

Compounds containing fatty acid residues appear to be especially susceptible. This applies to acetates, propionates, butyrates, caprylates, laurates, myristates, oleates and ricinoleates, the oils containing fatty acids such as tung, linseed, cottonseed, castor and dehydrated castor oils, and triacetin. Tung oil evidences slightly more resistance than do the other natural oils. Cyclohexyl lactate (a hydroxypropionic acid ester) was immune.

Esters of straight-chain dicarboxylic acids, while for the most part susceptible, seem slightly more resistant than do those of the monocarboxylic acids. Succinates, adipates, azelates, and sebacates are all attacked, although di-(2-ethylhexyl) azelate, the corresponding adipate, diethyl succinate, and methylcyclohexyl oxalate showed good resistance. A derivative of the hydroxylated dicarboxylic acid, di-n-butyl tartrate, was immune. Derivatives of the aliphatic tricarboxylic acids—aconitic, citric, and tricarballylic—appear to have good resistance, there being only a few instances in which they showed slight susceptibility. Conflicts in the data are evident.

Of the monocarboxylic aromatic acid derivatives, benzyl benzoate is attacked, whereas ethyl-*o*-benzoylbenzoate is not, and the terpenes—abietic acid and hydrogenated methyl abietate—show resistance. Of aromatic dicarboxylic acids, 24 phthalates were tested. For the most part they are immune, with the exceptions that there is some evidence of at least slight attack on diamyl-, diphenyl-, ethyl-2-methyl-2-nitro-propyl-, dibenzyl-, butyl-, dicapryl-, dimethyl-cyclohexyl-, and methyl phthalates. The general consensus is that phthalates as a class are resistant.

Fifteen organic phosphates were among the compounds tested. Of these the majority appeared to be resistant. Notable exceptions were diphenyl mono-*o*-xenyl-, diphenyl mono-(*p-tert.*-butylphenyl)-, and triphenyl phosphates and the tertiary phosphate of 2-nitro-2-methyl-propanol. Phosphates as a class are also considered to have satisfactory resistance (96).

Glycerol, pentaerythritol, and sorbitol derivatives, of which some 18 were included, for the most part appear to support from slight to luxuriant growth. Exceptions are di-pentaerythritol hexaacetate and the corresponding hexabutyrate, neither of which supported growth.

TABLE 1

RESISTANCE OF PLASTICS TO ATTACK BY MICROORGANISMS

Material	*Resistance*	*References*
Acrylics		
Polymethylmethacrylate	Good	13, 21, 31, 92
Polyacrylonitrile (Orlon)	Good	26, 37, 61
Acrylonitrile-vinyl chloride copolymer (Dynel)	Good	61, 68
Cellulose Derivatives		
Cellulose acetate*	Good, poor	6, 11, 13, 31, 46, 59
Cellulose acetate-butyrate	Good	13, 92
Cellulose acetate-propionate	Good	13, 92
Cellulose nitrate	Poor	13, 46, 92
Ethyl cellulose	Good	13, 92
Rayons		
Acetate Rayon (Estron)	Good	59, 61
Saponified Acetate Rayon	Slightly more resistant than cotton	59, 61
Cuprammonium Rayon	Poor	59, 61
Viscose Rayon	Poor	61
Phenol-Formaldehydes		
Phenol-formaldehyde**	Good, poor	13, 21, 96
Phenol-aniline-formaldehyde	Poor	13
Resorcinol-formaldehyde	Good	61
Melamine-Formaldehydes		
Melamine-formaldehyde†	Good, poor	13, 92, 99
Urea-Formaldehydes		
Urea-formaldehyde†	Good	13, 21, 92, 99
Protein-Formaldehydes		
Zein-formaldehyde (Vicara)	Good	61, 87, 101
Casein-formaldehyde	Poor	21

* Fully acetylated cotton is resistant, but there are acetylated cottons in which the percentage acetate is not high enough to impart complete resistance.

** Phenol-formaldehydes are probably resistant, and the discrepancy in opinions probably arises from testing samples containing susceptible fillers.

† White & Siu (99) in tests on cotton fabrics impregnated with urea-formaldehyde and melamine-formaldehyde resins found that a high degree of fungal resistance was imparted to the cotton by the resins. It was not conclusively shown, however, whether the resistance was due to the resins as such or to the possible presence of free formaldehyde.

TABLE 1—(*continued*)

Material	*Resistance*	*References*
Polyamides		
Nylon††	Good	6, 11, 21, 31, 59, 61, 96
Polyesters		
Ethylene glycol terephthalate (Terylene) (Fiber V)	Good	17, 35
Polyethylenes		
Polyethylene‡	Good	9, 13, 46, 92
Polytetrafluoroethylene (Teflon)	Good	13
Polymonochlorotrifluoroethylene (Kel-F)	Good	28, 46
Polyisobutylene	Good	21, 46
Styrenes		
Polystyrene	Good	13, 21, 31, 92
Polydichlorostyrene	Good	13
Vinyls and Vinylidenes		
Polyvinyl chloride	Good	9, 13, 21, 31, 92
Polyvinyl acetate	Poor	13, 92
Polyvinyl chloride-acetate	Good	13, 21, 31, 92, 96
Polyvinylidene chloride	Good	13, 21, 61
Polyvinyl butryal	Good	13, 96
Glyptal Resins	Poor	21
Silicone Resins	Good	13

†† Some tests have indicated nylon to be attacked in soil burial (22), but most evidence shows it to be immune.

‡ Klemme & Watkins (46) in 1950 reported that the susceptibility of polyethylene and polyisobutylene resins to fungus growth decreases as the average molecular weight increases. Ethylene materials of molecular weights above 10,000, and a butylene sample of 100,000 mol. wt., were found to be fairly resistant. Polytrifluorochloroethylene (Kel-F) shows a nutritive inertness comparable to the high-molecular-weight polyethylenes.

Glycol derivatives vary in resistance, depending upon the substituent part of the molecule. Diethylene glycol ethyl ether acetate and the corresponding butyl ether acetate, ethylene glycol laurate, ethylene glycol ethyl ether laurate, diethylene glycol monolaurate, diethylene glycol ethyl ether laurate, ethylene glycol methyl ether oleate, ethylene glycol methyl ether acetyl ricinoleate, diethylene glycol ethyl ether stearate, and tetraethylene glycol monostearate and distearate all evidenced moderate to heavy growth. On the other hand, diethylene glycol itself, dimethyl glycol

phthalate, diethylene glycol dipropionate, triethylene glycol di-(2-ethylhexoate) or the polyethylene compound, triethylene glycol di-(2-ethylbutyrate), the diacetate and dipropionate of 2-nitro-2-methyl-1,3-propanediol, and several polymers of ethylene glycol all showed good evidence of strong resistance. Glycolic acid derivatives, of which four were tested, all proved to be slightly to moderately susceptible. It appears that glycol and glycolic acid derivatives containing aliphatic chains no longer than 10 carbon atoms are resistant.

Of the remainder of the compounds tested, the chlorinated hydrocarbons, a silicone oil, two toluenesulfonates, *o*- and *p*-toluene ethylsulfonamide, a sulfonated oil, a coal-tar oil, petroleum oil, diphenyl, diamylnaphthalene, diamylphenoxyethanol, benzophenone, methylamyldihexylcyclohexanone, diphenyl sulfone, triphenylguanidine, butyraldehyde, chlorowax, decalin (hydrogenated naphthalene), ethyl acetanilide, and butadiene-acrylonitrile all appeared not to support fungal growth (8, 13, 92).

Among miscellaneous compounds which did support fungal growth were a refined tall oil, a nitrile from oleic and linoleic acids, a glycol sebacate resin, several sebacic acid alkyd resins, an ester-type alkyd resin, a fatty acid dimethylamide, and a triethanolamine dicaprylic acid salt.

Rubber

Although it is the common opinion that rubber, natural and synthetic, is not attacked by microorganisms, there is ample evidence that no categorical statement to that effect may be made. Some types of natural rubber or synthetic elastomers are attacked, while others appear entirely immune. The susceptibility appears to be a function not only of the predominating elastomer but also of the compounding materials in the finished product. The importance of deterioration of rubber by microorganisms is difficult to assess. Reactions are slow and evidences of attack are usually difficult to identify clearly in differentiation from attack on other substances which may be present.

On the susceptibility of natural rubber and synthetic elastomers to attack by microorganisms there have been several reports, in some of which summaries are given of previous work. Among these are the papers of Zobell (102, 103); Blake & Kitchin (9); Blake, Kitchin, & Pratt (10); and Greathouse (32). Zobell (102) points out that, although caoutchouc (natural rubber) is produced by many plants, it never accumulates in nature, thus suggesting that it undergoes decomposition. He further states that it is quite unstable, being easily oxidized in the presence of atmospheric oxygen, and that oxidation appears to occur especially readily under environmental conditions conducive to the growth of microorganisms. Rubber stoppers, pure gum rubber, and neoprene, according to Zobell, increased the oxygen consumption of bacteria in sea water.

Zobell & Beckwith (103) also found that rubber-destroying ability is not universal but showed that mixed cultures of soil bacteria will attack highly purified caoutchouc, neoprene, butaprene, chemigum, ameripol, hycar,

thiocol, butyl rubber, and several experimental elastomers synthesized from butadiene, isoprene, isobutylene, and acrylonitrile or styrene. Englerth (27) found two types of rubber gaskets to be attacked by a spore suspension of mixed fungi, although of the two the one made of reclaimed Buna S was attacked considerably more than the other made of new Buna S. An anonymous report (60) states that of six rubbers tested—two natural rubber compounds and four synthetic—all cured compounds were visually immune to the four sets of conditions of flask-wick nutrient-salts exposure for two weeks, filter-plate nutrient-salts exposure for two weeks, mold-cabinet spore-charged humid-atmosphere exposure for one month, and soil burial for three weeks. Of the uncompounded identical rubbers, neoprene was the only one resistant to mold growth, whereas the others were listed in order of decreasing resistance as Hycar OR-15, GR-I (butyl rubber), smoked sheet, pale crepe, and GR-S.

Table 2 indicates the susceptibility of various types of natural and synthetic rubbers to microorganisms, but a glance will show that much conflicting data exist. Since the data obtained are from several reports, the ambiguity undoubtedly arises from differences in test methods, in interpretation of results, and in the presence in the rubbers of various nutrients for microorganisms. Some data are available, as in an anonymous report in the "Vanderbilt News" (60) and in Blake *et al.* (10), where the distinction between attack on natural and on synthetic rubbers was made by visual inspection.

While such observations are perhaps indicative, they cannot be taken as conclusive since growths may be due to surface contaminants or to nutrients in the compounded rubbers. If one is interested in determining whether the growth of organisms is at the expense of rubber hydrocarbons, more searching criteria should be employed. Zobell and co-workers (102, 103, 104) and Blake and co-workers (9, 10) have demonstrated methods of making such critical evaluations.

Zobell *et al.* (102, 103) used oxygen consumption, carbon dioxide production, multiplication of microorganisms, and deterioration of the rubber as criteria. Using these observations, they have stated that pure natural rubber, compounded rubber products, and several of the synthetic elastomer hydrocarbons—notably polychloroprenes, butadiene-styrenes, butadiene-acrylonitriles, organic polysulfides (thiokols), and isoprene-isobutylenes—are attacked by mixed cultures of soil bacteria. Zobell states that most uncured synthetic rubbers are very unstable and that even when compounded with preservatives they appear to be attacked by bacteria. He also states that polysulfide rubbers (thiokols, types A and B), vinylites, and chlorinated rubbers appear relatively resistant to bacterial attack, but these products have few of the characteristics of live rubber.

Zobell further generalizes that unsaturated hydrocarbons are attacked more readily than saturated compounds, long chains more readily than short. Attack also seems to be a function of the number of side chains, *iso* compounds generally being more easily attacked than normal hydrocarbons.

TABLE 2

RESISTANCE OF NATURAL AND SYNTHETIC RUBBERS TO MICROORGANISMS

Material	*Resistance*	*References*
Natural Rubber		
Pure natural rubber—caoutchouc	Attacked	9, 10, 102, 103
Highly purified natural rubber, 99%+, not vulcanized	Attacked	103, 104
Natural rubber vulcanizate	Attacked	9, 24, 104
	Resistant	85
Hevea latex	Attacked	103
Guayule latex	Attacked	103
Crude sheet	Attacked	21, 103
Crepe rubber	Attacked	103
Pale crepe, not compounded	Attacked	9, 60
Pale crepe, compounded	Resistant	60
	Attacked	9
Plantation crepe	Attacked	103
Smoked sheet, not compounded	Attacked	9, 60
Smoked sheet, compounded	Resistant	60
	Attacked	9
Reclaimed rubber	Attacked	25
Gutta-percha	Some attack but less than natural rubber	9
Chlorinated rubber	Resistant	103
Synthetic Rubbers		
Neoprene-polychloroprene, not compounded	Resistant	24, 25, 60
	Attacked	102, 103
Neoprene, compounded*	Resistant	9, 21, 24, 60, 85
	Attacked	10, 104
GR-S, butadiene-styrene, not compounded	Resistant	9, 10
	Attacked	60
GR-S, butadiene-styrene, compounded	Resistant	60, 85
	Attacked	9
GR-S, butadiene-styrene, compounded, acetone extracted	Resistant	10
Buna-S, butadiene-styrene, uncured	Attacked	103
Hycar OR, butadiene-acrylonitrile, not compounded	Attacked	21, 60, 102, 103
Hycar OR, butadiene-acrylonitrile, compounded	Resistant	60
	Attacked	21
Buna N, butadiene-acrylonitrile, compounded	Attacked	24
GR-I (Butyl), isobutylene-isoprene, uncured	Resistant	9, 10
	Attacked	60, 102, 103

* Neoprene containing nutrients may be attacked, but the hydrocarbon itself is not attacked (10).

TABLE 2—(*continued*)

Material	*Resistance*	*References*
GR-I (Butyl), isobutylene-isoprene, compounded	Resistant	60
	Attacked	9
Thiokol, organic polysulfide, uncured	Attacked	102, 103
Thiokol, organic polysulfide, vulcanized	Resistant	21, 103
Thiokol, organic polysulfide, sheets for gasoline tank linings	Attacked	2
Experimental elastomers from:		
Butadiene	Attacked	102
Isoprene	Attacked	102
Isobutylene	Attacked	102
Acrylonitrile	Attacked	102
Styrene	Attacked	102

He likewise states that pure rubber is oxidized by bacteria more rapidly than compounded or vulcanized products, the rate of oxidation depending upon the amount of surface exposed to attack. Yet, he points out, neither in nature nor in the laboratory do rubber-oxidizing bacteria appear to require rubber hydrocarbons for growth. Though most abundant on moist rubber, rubber-oxidizing bacteria have been demonstrated in nearly all samples of marine mud, sea water, sewage, tap water, and soil which have been examined for their presence. Old pieces of garden hose and the cracks in rubber products left in moist places have proved to be good sources of cultures.

It should not be interpreted that biological degradation of rubber is a rapid reaction. As Zobell points out, even in the presence of good nutrient solution, the rubber is only slowly attacked. It is decomposed more rapidly by mixed than by pure cultures (103).

Blake *et al.* (9, 10) using electrical resistance measurement tests of wire insulated with natural rubber, various synthetic rubbers, and plastics, and supplementing these observations with electrolytic copper depositions and hydrogen evolution through micropores caused by bacterial attack, as well as by color indicators and d-c corona tests for these same faults, came to the conclusion, in contradistinction to Zobell *et al.*, that although natural rubber hydrocarbon is attacked, the base hydrocarbons of several synthetics are not. They state that natural rubber insulation breakdown is accompanied by visible pitting, whereas synthetic rubber insulation breakdown is not. They attribute the observed irregularly shaped micropores to attack by organisms on the nutrient materials present in the synthetic rubber compositions. They are able to say, therefore, that GR-S, butyl, and neoprene, as such, are not attacked.

It thus appears, in a brief glance at the entire picture, that there is more or less complete agreement that natural rubbers, pure or compounded, are

susceptible to attack by microorganisms. On the synthetic rubber side, the situation is a little ambiguous, some synthetics apparently being of undoubted resistance, others of questionable resistance. It further appears that not only should the base hydrocarbon of the synthetic be examined, but also the composition of the entire specific synthetic compound should be considered.

Hydrocarbons

There seems to be little doubt that microorganisms attack many hydrocarbons. Some everyday examples of this attack are the decomposition of cooling-oil emulsions, deterioration of oiled or asphalt-surfaced highways, and the modification of petroleum or its products stored in the presence of water (97, 102). It is difficult to assign a monetary value to such losses, but it is likely that such an estimate would be high.

The general field of hydrocarbon susceptibility to microorganisms has been reviewed up to 1946 by Zobell (102). He states that all types of gaseous, liquid, and solid hydrocarbons in the aliphatic, olefinic, aromatic, and naphthenic series appear susceptible to oxidation by microorganisms if the compounds are properly dispersed. Aliphatic compounds are attacked more easily than aromatic and naphthenic compounds and, within certain limits, long-chain hydrocarbons are attacked more readily than similar compounds of small molecular weight. Addition of aliphatic side chains increases the susceptibility of cyclic compounds.

Zobell lists the following organisms as able to assimilate one or more kinds of hydrocarbons: 14 species of *Actinomyces*; 13 *Pseudomonas*; 10 *Proactinomyces*; 10 *Mycobacterium*; 9 *Bacillus*; 7 *Bacterium*; 7 *Micrococcus*; 6 *Aspergillus*; 5 *Corynebacterium*; 3 *Vibrio*; 2 *Achromobacterium*; several unidentified strains of *Micromonospora* and *Penicillium;* and one or more strains of *Botrytis*, *Debaromyces*, *Desulfovibrio*, *Endomyces*, *Escherichia*, *Gaffkya*, *Hansenula*, *Macromonospora*, *Methanomonas*, *Mycoplana*, *Serratia*, *Spirillum*, *Torula*, and *Torulopsis*.

Characterizing as "antiseptic" the hydrocarbons such as benzene, toluene, xylene, naphthalene, and related compounds, Zobell states that they are destroyed by soil microorganisms. Crude oils, illuminating gases, petroleum ethers, gasolines, kerosenes, fuel oils, paraffin or mineral oils, petrolatums, asphalts, paraffin waxes, as well as numerous chemically pure hydrocarbons, have, over a period of many years and by numerous investigators, been shown to be oxidized by a large variety of microorganisms, as generalized by Zobell. He warns, however, that results attributable to particular hydrocarbons may possibly be due to other types of organic or inorganic compounds present, or to peculiar experimental conditions.

In 1942 Johnson, Goodale & Turkevich (42) found that *Bacterium aliphaticum* Tausz on a simple inorganic medium will readily use pentane, hexane, heptane, octane, nonane, dodecane, gasoline, tetraethyllead gasoline, and lubricating oil and to a slight extent *iso*-octane and methyl cyclohexane but not benzene, toluene, or xylene. In 1946, Turkevich & Hiller (90) determined that *B. aliphaticum* will not attack preferentially one of the two

optical isomers of 3-methylhexane or of 3-methylheptane. This investigation was evidently made in the hope that such a culture might be used for separating isomers. Rosenfeld (73) in 1947 reported that *Desulfovibrio* species catalyze the anaerobic oxidation of a considerable variety of mixed hydrocarbons, and the activity appears to be associated with the presence of a dehydrogenase system.

In an investigation of the mechanism of attack on paraffins by microorganisms, Baldacci (3) in 1947 found that, although several aliphatic saturated and unsaturated monobasic acids were attacked, cyclohexanone was not. Commercial paraffin, however, supported considerable growth. Osnitskaya (66) in 1947 found a pure culture of an aerobic microorganism capable of oxidizing 90 per cent of a naphthenic acid in 10 days. Mogilevskii & Dianova (62) in 1947 were issued a Russian patent for analyzing hydrocarbon and similar gases by means of bacterial cultures with ability to oxidize definite components of the gas.

Kuznetsov (47) studied several Ural lakes to determine the presence of bacteria capable of oxidizing hydrocarbons and found that fresh-water lakes usually contain such organisms, although sometimes in small concentrations. He found that the silt deposits contain a large number of oxidizers of hydrogen, methane, naphthalene, and phthalic acid, while hexane and heptane oxidizers were less common and oxidizers of toluene and propane were rare.

Imelik (39) in 1948 inoculated with soil a petroleum medium containing 0.1 per cent ammonium chloride and in four weeks found increased naphthenic acids, partial disappearance of the lighter fraction of the aromatic and ethylenic hydrocarbons, conversion of saturated to unsaturated hydrocarbons, and disappearance of considerable amounts of cyclic hydrocarbons. He, like others, suggests that bacterial action offers a possibility for cracking heavy hydrocarbons and particularly for opening rings.

Imelik (40) also found satisfactory growth of *Pseudomonas aeruginosa* in a mineral medium containing petroleum ether, heptane, gas oil, or petrolatum. In 1949, the same investigator (41) studied the enzymatic mechanism of oxidation of hydrocarbons by bacteria and showed that with *P. aeruginosa* inorganic phosphate is not necessary for hydrocarbon oxidation. He characterizes the enzymes which act on hydrocarbons as constitutive and states that they are inhibited by potassium cyanide and urethane, indicating a dehydrogenase type of activity.

Metals

According to Uhlig (91), the cost of underground pipe maintenance and replacement in the United States is 600 million dollars per year. The action of microbiological agents contributes significantly to this corrosion bill.

The subject of corrosion of metals by microorganisms was reviewed by Hadley (36) in 1948. He defines microbiological corrosion as the deterioration of a metal by corrosion processes which occur, either directly or indirectly, as a result of the metabolic activity of microorganisms. He differentiates between microbiological corrosion and the microbiological fouling frequently

found in water pipes and on metallic surfaces exposed to miscellaneous aqueous environments. According to Hadley, microorganisms contribute to corrosion by one or more of the following means: direct influence on the rate of anodic or cathodic reaction, change of surface metal film resistance by metabolism or products of metabolism, creation of corrosive environment, and establishment of a barrier by growth and multiplication so as to create electrolytic concentration cells on the metal surface.

Rogers (72), in a similar approach, more specifically attributes the direct action to the production of corrosive substances such as carbon dioxide, hydrogen sulfide, ammonia, or organic or inorganic acids. For indirect action he suggests metabolic products acting as depolarizers or catalysts of corrosion reactions, and in the third classification he lists cases similar to anaerobic corrosion of iron by the activities of the sulfate-reducing organisms where, under some conditions, the corrosion reaction is an integral part of the metabolic cycle of the bacteria.

Hadley classifies corrosion-causing organisms as anaerobic (sulfate-reducing vibrios, nitrate-reducing microorganisms, and the methane-producing bacteria) and aerobic (sulfur bacteria including thiobacilli; the iron bacteria; and miscellaneous organisms including slime-forming bacteria, fungi, algae, protozoa, diatoms, and bryozoa). He states that, of the many microorganisms associated with microbiological corrosion, the sulfate-reducing bacteria of the type species *Sporovibrio desulfuricans* (Beijerinck) Starkey have the most prominent role, both with regard to prevalence and to economic importance. The sulfate-reducing bacteria were shown in 1949 to be important not only in external corrosion of pipes but in internal as well (16).

Glass

That glass is deteriorated through the action, direct or indirect, of microorganisms appears to be quite firmly established (38, 43, 93). Although the actual extent of such action may be only slight, the nature of the material and the uses to which it is put make even slight damage important. Lenses and other glass parts of optical and photographic equipment constitute some of the most important and most costly equipment used by many industries and certainly by the Armed Forces. Even slight fogging or etching of such parts may render an instrument unusable.

Hutchinson (38) states that many of the types of damage associated with the presence of microorganisms on glass surfaces may be induced in the absence of the organisms, but it is considered that damage to glass surfaces is made more rapid by the metabolic products of microorganisms. He further states that the so-called "channeling" of glass surfaces represents a type of damage unique to fungus infestation. The active presence of the microorganisms on the glass surfaces may be attributed to the presence of organic contaminants, such as dead bodies of mites or other small insects and spiders, or of other nutrient materials. Channeling or other types of etching may be so evident as to be detected by rubbing a needle over the surface. Fogging or clouding, although of less penetrative nature, is observable visually.

According to Jones (43), fungi produce organic acids that cause etching of glasses subject to acid attack. On alkali-lime-silicate glasses, fungi sometimes produce alkali-type etching by maintaining the correct water supply to produce a concentrated solution of sodium or potassium carbonate.

Conclusion

In general summary, then, it appears that microbiological attack on manufactured materials is far from uncommon. All kinds of basic structural materials—leather, plastics, textiles, wood and wood products, and even the surfaces of materials often thought to be adequately protected by paint—are subject to microbiological invasion with concomitant deteriorative effects. The incorporation of such materials into assemblages, especially if not treated to resist microorganisms, literally invites infestation if only because of the diversified selection of nutrients thus afforded.

Both organic and inorganic materials appear susceptible, the difference in behavior between the two classes resting in the mode and severity of attack. While organic materials furnish the most attractive nutrients for microorganisms, some inorganic materials and free metals supply the necessary mineral component of the substrate. As might be expected from the general characteristics of nature, organic substances as a class are more easily and more generally susceptible, but the biological deterioration of inorganic materials nevertheless poses difficult scientific and economic problems. The organisms responsible for the defacement or destruction of materials make up a complete, or almost complete, biological spectrum. Bacteria, fungi, actinomycetes, yeasts, algae, and protozoa all seem to play a part.

Although considerable progress has been made in gathering factual knowledge on the microbiological susceptibility of many manufactured materials, present knowledge of the mechanism of action of microorganisms on these materials is still fragmentary. Because of the twofold importance of the subject—economically, and with respect to conservation of natural resources—there seems little doubt as to the need for more fundamental work in this interesting and promising field.

Acknowledgment

The authors acknowledge with grateful thanks the assistance of the members of the staff of the Prevention of Deterioration Center in the preparation of this review.

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